THE UNIVERSITY OF ALBERTA

DEVELOPMENT OF LIPOSOMAL ANTIGEN DELIVERY SYSTEM FOR SYNTHETIC MUC1 PEPTIDES

BY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled:

DEVELOPMENT OF LIPOSOMAL ANTIGEN DELIVERY SYSTEM FOR SYNTHETIC MUC1 PEPTIDES hereby submitted by Holly Guan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences

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Dedicated

to

My Dear Parents, Professor Yi Guan and Nailan Wang

Abstract

Synthetic MUC1 peptides are potential targets for active specific immunotherapy of cancer. This thesis describes the development of a liposomal delivery system for synthetic MUC1 peptides favoring type 1 immune responses. A high performance liquid chromatographic methodology was developed to quantify MUC1 peptides in liposomes. Encapsulation efficiencies of hydrophilic MUC1 peptides were significantly improved in charged liposomes under low ionic strength conditions. More than 75 percent of MUC1 peptides were retained in liposomes during 37°C incubation in 100 percent mouse or human serum for 24 h, and 85 percent were retained at 4°C in PBS for at least 12 months. Liposomal formulations were characterized for surface expression of MUC1 peptide epitopes. Liposomes encapsulating N-terminal acylated MUC1 peptide, but not unmodified MUC1 peptide or C-terminal acylated peptide, showed MUC1 epitopes on the liposome surface. The nature of MUC1 peptide formulation containing monophosphoryl lipid A (MPLA) profoundly influenced both cellular and humoral immune responses. Strong and MUC1-specific T cell proliferative responses were elicited by liposome-associated rather than non-associated peptides, suggesting a requirement of peptide association with liposomes for effective delivery to antigen presenting cells and induction of T cell responses. The exact location of the peptide in liposomes was not critical for the type and magnitude of T cell

responses. Both masked and surface-exposed peptide liposome formulations elicited strong and MUC1-specific T cell responses, accompanied with high levels of IFN-γ, but not IL-4. However, the location of peptide in liposomes significantly influenced the humoral immune response. Only surface-exposed MUC1 liposomes induced high levels of anti-MUC1 antibodies dominated by the IgG2b isotype. Over the dose range of the peptide tested, human MUC1 specific T cell responses did not exhibit dose-dependency in BALB/c or C57BL/6 mice. The effect of phospholipid composition and size of liposomes on T cell responses was studied using a mouse MUC1 peptide (tandem repeat consensus sequences) as the antigen. This peptide induced antigen-specific T cell responses in a dose-dependent fashion (range 12-100 μg per mouse) in BALB/c mice. This homologous T cell response was independent of phospholipid composition and liposome size. In summary, strong and MUC1 specific type 1 associated immune responses were elicited by MPLA containing MUC1 liposomes. A liposomal delivery system can be tailored to preferentially induce cellular and/or humoral immune responses. The results of this study are expected to advance knowledge in vaccine design.

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List of Abbreviations

A	Alanine
Ab	Antibody
ADCC	Antibody dependent cell-mediated cytotoxicity
Ag	Antigen
APCs	Antigen-presenting cells
ASI	Active specific immunotherapy
AUP	Area under peak
BCA	Bicinchoninic acid
BGG	Bovine γ-globulin
BSA	Bovine serum albumin
C	Cysteine
CD	Cluster of differentiation
CHOL	Cholesterol
CPM	Counts per minute
CTL	Cytotoxic T lymphocytes
Cys	Cystine
D	Aspartic acid
DCP	Dicetyl phosphate
ddH_2O	De-ionized distilled water
DMPC	Dimyristoyl phosphatidylcholine
DMPG	Dimyristoyl phosphatidylglycerol
DPPC	Dipalmitoyl phosphatidylcholine

DSPC	Distearoyl phosphatidylcholine
DTH	Delayed-type hypersensitivity
E	Glutamic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FAT	Freeze and thaw
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G	Glycine
g	Gravity
H	Histidine
h	Hour(s)
HPLC	High performance liquid chromatography
HSA	Human serum albumin
I	Isoleucine
i.e.	For example
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IL-4	Interleukin 4
IL-12	Interleukin 12
i.p.	Intraperitoneal
i.v.	Intravenous
ISCOMS	Immunostimulating complexes

KLH	Keyhole limpet hemocyanin
l	Liter(s)
LPS	Lipopolysaccharide
Lys	Lysine
M	Molar
mAb	Monoclonal antibody
mon	Month(s)
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
min	Minute(s)
MLVs	Multilamellar vesicles
µCi	Microcurie(s)
µg	Microgram(s)
µm	Micrometer(s)
MLVs	Multilamellar vesicles
mm	Millimeter
mM	Millimolar
MPLA	Monophosphoryl lipid A
MTP-PE	Muramyl tripeptide-phosphatidylethanolamine
N	Asparagine
ng	Nanogram(s)
nm	Nanometer(s)
nmole	Nanomole(s)

NK	Natural killer cells
O.D.	Optical density
OVA	Ovalbumin
o/w	Oil-in-water
P	Proline
PBS	Phosphate buffer solution
pI	Isoelectric point
PS	Phosphatidylserine
PSA	Prostate specific antigen
Q	Glutamine
QS21	Quillaja saponin 21
R	Arginine
RES	Reticuloendothelial system
RT	Retention time
rIFN-γ	Recombinant gamma interferon
rIL-4	Recombinant Interleukin 4
rpm	Rotations per minute
S	Serine
SA	Stearylamine
s.c.	Subcutaneous
S.I.	Stimulation index
T	Threonine
Tc	Phase transition temperature

TCR	T cell receptor
TEA	Triethylamine
TFA	Trifluoroacetic acid
Th	T-helper
TPBS	Tween 20 phosphate buffer solution
TT	Tetanus toxoid
UV	Ultraviolet
V	Valine
v	Volume
w	Weight

I. INTRODUCTION

A. Vaccine Design

A vaccine is a nonpathogenic immunogen that, when inoculated into a host, induces protective immunity against a specific pathogen. Vaccines rank among the most important contributions of science to human health, and the developments of the first vaccines against smallpox by Edward Jenner in 1798 and rabies by Louis Pasteur in 1880 are landmarks in the history of immunology. Effective vaccination programs have drastically reduced the incidence of many infectious diseases that once were common. As a result, smallpox was completely eradicated as a human disease after the last case was reported in 1977 (1). Understanding the mechanisms employed in induction and maintenance of immune response may aid in our ability to manipulate the response and to develop strategies for effective vaccines.

1. Characterizations of Immune Responses Relevant to Vaccine Development

a. cellular and humoral immunity

The immune system can be divided into two broad functional categories: humoral immunity and cellular immunity. Humoral immunity is effected by B cell produced antibodies, bivalent protein molecules that can be cell-bound or cell-free. Antibodies bind to antigenic determinants,

usually on the surface of bacteria or parasites, and inactivate these infectious agents. Antibodies are primarily effective against extracellular pathogens. By contrast, cellular immunity is mediated by effector cells such as natural killer cells (NK) and $CD8^+$ cytotoxic T lymphocytes (CTL), that destroy the infected cells or tumor cells by direct cell-to-cell contact, or by the release of molecules that posses killing activity (2). In this way, cellular immune mechanisms focus on destruction of the source of infection or alteration and are more effective against intracellular diseases.

However, the split between humoral and cellular immunity is not definitive and interactions between them are required for the induction of fully functional immune responses. If antibody binds to a target cell, for example, a tumor cell or a virus infected cell, it may activate the complement system through the classical pathway, or it can sensitize the cell for attack by cytotoxic cells expressing Fc receptors. This antibody dependent cell-mediated cytotoxicity (ADCC) is performed by NK cells and some activated macrophages. These cells elicit damage by releasing enzymes, cytolysins, reactive oxygen intermediates and cytokines in proximity to the target. ADCC plays an essential role in modulating responses to virus infected cells (3).

The complement system is a group of serum proteins which may be activated by immune complexes via the classical pathway or by surfaces via the alternative pathway. Complement activation involves an

enzymatic cascade and both of the pathways converge in a common terminal reaction sequence that generates a membrane-attack complex (MAC) leading to membrane disassembly and osmotic lysis. Complement components and antibodies are also called opsonins which coat on antigens to facilitate the uptake by phagocytic cells.

b. antigen processing and presentation

Unlike B cells, that can recognize soluble and native antigens through specific antibody binding, T cells can only recognize processed antigens presented by major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (APCs). APCs serve at least two functions in T cell recognition (4). First, the APCs take up and process the antigens via phagocytosis or receptor mediated endocytosis. Second, APCs present the processed peptide bound in a groove on the MHC molecules to T lymphocytes. Three cell types are classified as professional APCs: dendritic cells, macrophages and B-lymphocytes, differing in the mechanisms of antigen uptake and expression of surface molecules involved in antigen processing and presentation. Receptor-mediated endocytosis is critical especially for nonparticulate antigens. B cell surface immunoglobulin (Ig) molecules can act as a specific high-affinity receptor to allow efficient uptake of antigen for its subsequent processing and presentation to T lymphocytes (5).

Class I MHC molecules bind peptides for presentation to cytotoxic T cells, the majority of which express the CD8 accessory molecules.

Class II MHC molecules bind peptides for presentation to T helper (Th) cells carrying the CD4 accessory molecule (6) (Figure 1).

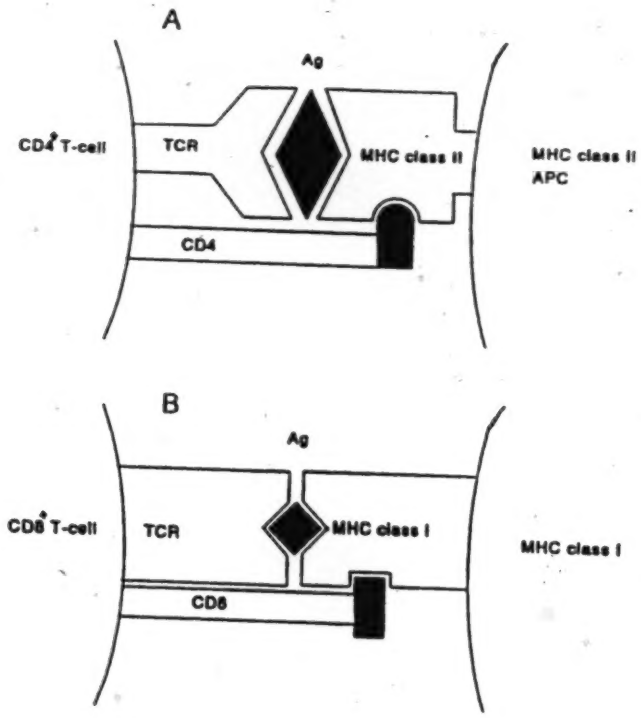

Figure 1. Schematic representation of antigen presentation to T cells. (A). An APC presents processed exogenous antigen as peptide-MHC class II complex to a specific T cell receptor (TCR) of a $CD4^+$ T cell. (B). A cell (not necessarily an APC) presents endogenous antigen as peptide-MHC class I complex to a specific T cell receptor of a $CD8^+$ T cell. (Based on reference 6).

Peptides presented by class I MHC molecules are generally 8-11 amino acids in length and contain specific contact amino acids for use as

anchor residues, which vary for different MHC allelic products. Peptides expressed in association with class II MHC molecules are typically longer, 12-25 amino acids in length, and they also appear to contain specific motifs that are preferentially bound by different class II MHC allelic products (7).

The peptides that are expressed in association with either the class I or II MHC molecules are generated through two different intracellular pathways. Soluble proteins and particulates are taken up by the exogenous pathway of APCs and presented in association with class II molecules; whereas antigens arising from cytosol are following endogenous pathway and presented in association with class I molecules (Figure 2).

Presentation of antigen by class I or class II MHC molecules is dependent on its route of introduction into the cell. In the exogenous pathway, the internalized antigen by phagocytosis and/or endocytosis is degraded into peptides via three increasingly acidic compartments: endosomes, endolysosomes and lysosomes. Cleaved peptides bind to class II MHC molecules and the peptide-MHC class II complex is transported on the cell surface for presentation to $CD4^+$ T helper cells (Figure 2). Class II MHC molecules are 'protected' from picking up peptides in the endoplasmic reticulum and Golgi complex by the invariant chain (8), which is subsequently removed by proteolysis, probably at the

same site where exogenous antigens are processed, allowing the class II molecule to bind peptide (9).

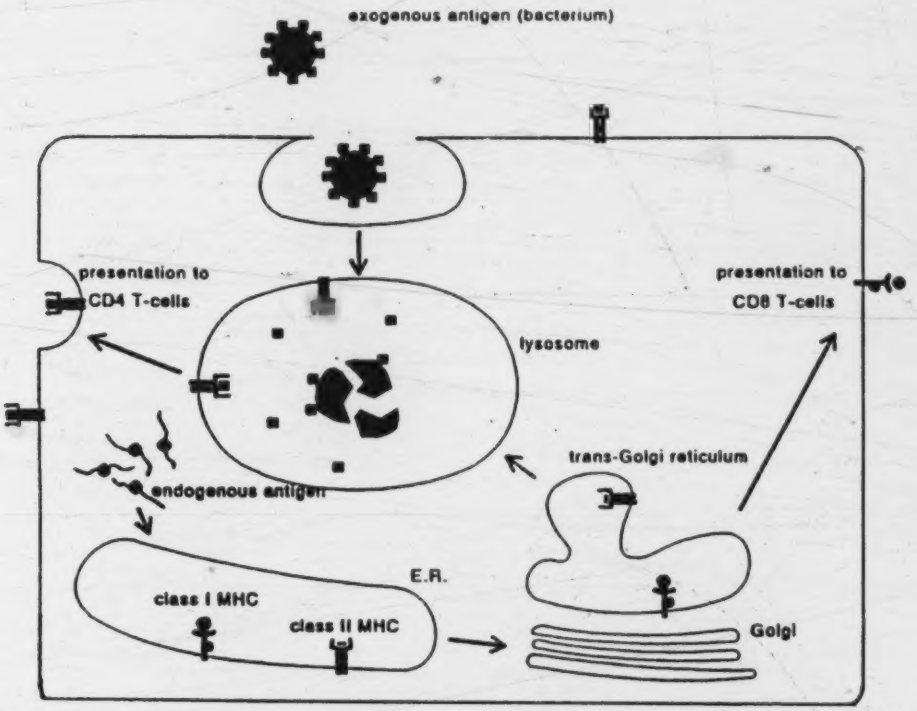

Figure 2. Schematic illustration of exogenous and endogenous pathways of antigen processing and presentation. Exogenous antigens are phagocytosed by phagocytic cells. In the late endosomes/lysosomes, enzymatic degradation takes place. The immunogenic peptides formed during the process of degradation bind to MHC class II molecules. Subsequently, the peptide-MHC class II complex is transported to the cell surface for presentation to $CD4^+$ Th cells. Antigens that are synthesized and/or processed in the cytoplasm bind to MHC class I molecules. The peptide-MHC class I complex is transported through the Golgi complex to the cell surface for presentation to $CD8^+$ T cells. MHC molecules are recycled through endosomal uptake. (Based on reference 6).

Proteolysis is also important for the presentation of antigens by class I MHC molecules, however, proteins degraded by the exogenous pathway are not usually presented by class I molecules (10, 11). Instead, most class I associated peptides result from proteins synthesized or introduced in the cytoplasm. These observations point toward the necessity of cytoplasmic degradation for peptide presentation by class I MHC molecules. In the endogenous pathway, proteins are degraded into peptides by a large multicatalytic proteolytic particle, the proteasome, and transported via the transporter associated with antigen processing (TAP) to endoplasmic reticulum, where the peptide binds selectively to class I MHC molecules. The peptide-MHC class I complex is transported and expressed on the cell surface for presentation to $CD8^+$ T cells (Figure 2). Exogenous and endogenous pathways of antigen processing are not completely discrete. There are examples of class II restricted T cells can recognize endogenous antigens (12), and class I restricted recognition of exogenous antigen (11).

Besides binding with peptide-MHC complex, differentiation of naive T cells into cytokine-producing effector Th cells requires a second signal, co-stimulatory signals exemplified by B7 (13). CD28 and CTLA-4 are B7 counterreceptors expressed on majority T cells.

c. T-helper (Th) subsets

Both cellular and humoral immunities require a special type of T cell, $CD4^+$ Th cells, to initiate and enhance the immune responses (14).

Th cells play a central role in regulating immune responses through cytokine secretion. Th cells assist in activation of B cells to mature into antibody secreting plasma cells, Ig class switching, activate phagocytic macrophages and promote the differentiation of pre-cytotoxic cells into competent killers. Two functionally distinct Th subsets in mouse are identified (15). Based on the cytokines they secrete, Th cells have been classified as Th1 and Th2. Th1 cells secrete IFN-γ, IL-2, but not IL-4 or IL-10; whereas Th2 cells secrete IL-4 and IL-10, but not IFN-γ or IL-2.

The same precursor T cells (Th0) can be developed into either Th1 or Th2 effector cells under the influence of a number of factors including type of adjuvant, cytokines in presence, co-stimulatory signals, antigen dose, the mode and route of administration (2, 16, 17). Although mechanisms controlling the type of Th cell differentiation remain elusive, it is believed that cytokines are the major factors determining the differentiation pathway of Th0 cells into Th1 or Th2 cells (14, 18). Role of antigen presenting cells (B cells and macrophages) in differentiation of effector Th populations from naive precursor T cells was studied *in vitro* by using peptide antigens (18). It was found that with both APC populations, effector Th cells developed in the presence of IFN-γ exhibited Th1 cytokine profile, whereas Th cells generated in the presence of IL-4 acquired Th2 cytokine profile and Th0 cytokine profile was observed in Th cells developed without IFN-γ or IL-4. It was also found that when recultured in the presence of IFN-γ or IL-4, the Th

effectors assumed a more Th1 or Th2-like phenotype, respectively, regardless of their initial cytokine profile (18). Presence of cytokines (IFN-γ or IL-4) dominated the development of Th effector subsets regardless of the APC populations.

IL-12 has been found a key regulating cytokine in the development of Th1 effector cells by stimulating their differentiation and proliferation (19). IL-12 is mainly secreted by activated macrophages and dendritic cells in response to bacterial or parasite infections. IL-12 induces proliferation of NK cells and Th1 cells resulting in increased IFN-γ production. Because IFN-γ activates macrophages, it has a positive feedback effect inducing more IL-12. It has been shown that IL-12 is an effective adjuvant by initiating protective $CD4^+$ Th1 cell-mediated immunities against leishmaniasis in BALB/c mice (20).

It seems to follow that the relative levels of IL-4 and IL-12 at the time of priming of naive T cells will determine the course of an immune response. The inducing effect of IL-4 dominates over other cytokines: differentiation of the Th0 cell into the Th2 phenotype occurs when IL-4 levels reach a necessary threshold (16). IL-4 and IL-12 are important as inhibitors of differentiation. IL-12 depresses IL-4 and IgE production, and IL-4 blocks the development of Th0 cells into IFN-γ precursors and this effect can be abrogated by simultaneous administration of IL-12. IL-12 and IL-4 are crossregulating one another's activities. This interplay may

result in a predominance of Th1 or Th2 cells following preferential stimulation of a particular Th subset.

Co-stimulatory molecules may influence T cell differentiation quantitatively, by determining the extent of T cell activation, and qualitatively, by influencing the cytokines that are produced by Th cells once they become activated (21, 22). It has been suggested that co-stimulatory molecules on APCs may selectively influence T cell differentiation: antibodies against B7-1 or B7-2 selectively inhibit the development of Th1 or Th2 responses, respectively, both *in vivo* and *in vitro* (23, 24). It was found that interaction of B7-1 or B7-2 with shared counterreceptors CD28 resulted in very different outcomes in clinical disease by influencing the differentiation of Th0 to a Th1 or Th2 lineage, respectively, through affecting the initial cytokine secretion. However, it is still controversial regarding the role of B7-1 and B7-2 on APCs, CD28 and CTLA-4 on T cells in the development of IL-4 producing T cells (22).

Th1 responses are normally characterized by cellular immunity and Th2 by humoral immunity. However, this is an oversimplification because not all humoral immune responses are enhanced by Th2 clones. Certain subclasses of IgG antibodies have been found associated with Th1 immune responses (25-27). Here are a few examples. Through *in vivo* study of influence of IL-12 on humoral immune responses, it was found that synthesis of antigen specific antibodies of IgG2a, IgG2b and IgG3 subclasses increased 10- to 1000-fold, whereas IgG1 not or only slightly

enhanced and IgE suppressed (26). In contrast, large amounts of IgG1 as well as some IgE but little IgG2a, IgG2b and IgG3 antibodies were developed in mice immunized with the same antigens but without coadministration of IL-12. In addition, the effects of IL-12 *in vivo* were found at least partially IFN-γ dependent because an anti-IFN-γ mAb in combination with IL-12 prevented most of the enhanced IgG2a productions (26). Another study demonstrated, via cytokine knockout mice, that IFN-γ -/- mice produced significantly reduced IgG2a and IgG2b antibodies, but maintained comparable levels of IgG1 and IgE compared to the cytokine-intact controls; in contrast, IL-4 -/- mice showed significantly decreased IgG1 and IgE serum immunoglobulins, but maintained comparable levels of IgG2a and IgG2b antibodies (27).

Contrary to the above two examples, other studies have found that IgG2b subclass antibody is associated with Th2 type immune responses. For example, when mice were given ovalbumin (OVA) vaccine formulation subcutaneously, high-titered serum anti-OVA IgG1 and IgG2b antibodies were developed (28). Quantitative analysis of cytokines secreted by the induced OVA-specific $CD4^+$ T cells revealed that Th2 type immune responses had been elicited. Thus, it is still not clear whether IgG2b responses are associated with type 1 immune responses or not.

2. The Role of Adjuvants in Modulation of Immune Responses to Vaccines

Traditionally, adjuvants have been used with routine human vaccines to elicit high and long lasting immune responses. In recent years, adjuvants have received much attention because of 1) poor antigenicity of newly developed purified subunit and synthetic vaccines which need adjuvant to evoke the immune response, and 2) selective modulation of immune response by adjuvants regarding types of antibodies, Th cells and MHC molecules (29).

Adjuvants have been used to modulate the response to different antibody isotypes to vaccine antigens (30). Cholera toxin, an oral adjuvant, modulated immune response to elicit IgA, IgG and IgE antibodies, whereas delivery of antigen by a live vector elicited mainly IgG antibodies (31). Antigens injected with alum mainly elicited IgG1 antibodies, but those injected with stearyl tyrosine elicited relatively higher levels of IgG2a and IgG2b antibodies in mice (32).

It is recognized that adjuvants modulate the immune response to different Th types. Several adjuvant formulations including lipopolysaccharide (LPS), monophosphoryl lipid A (MPLA), liposomes, muramyl dipeptide (MDP), and QS21 have been shown to preferentially stimulate Th1 type immune response in mice, accompanied by IFN-γ leads to cell-mediated immune response and production of relatively high levels of IgG2a antibodies in mice (33, 34). However, alum and cholera

toxin as mucosal adjuvants are prone to stimulate type 2 immune response accompanied by IL-4 and IL-10 leads to production of IgG1 and IgE antibodies in mice (31, 35).

Adjuvants can also modulate antigen processing and presentation, and consequently, lead to vaccines which can elicit Th and/or CTL responses. An MHC class I response usually appears after infections with intracellular pathogens such as viruses, leading to a CTL production as followed the endogenous pathway. However, by using adjuvants such as liposomes and immunostimulating complexes (ISCOMS), CTL responses have been elicited to exogenous peptide and protein antigens (36, 37).

The emergence of MPLA as a promising adjuvant for human vaccines can be traced back to 1956 (38). Johnson *et al* reported that endotoxin (LPS) derived from Gram-negative bacteria was a potent adjuvant for protein antigens. However, LPS has not found use as an adjuvant in vaccines except for experimental investigations. The primary reason is the toxicity associated with it. Many attempts were made to modify LPS so that its toxicity could be attenuated while retaining its immunostimulatory activities. These efforts were largely unsuccessful until it was discovered that a by-product, obtained during mild acid hydrolysis of LPS to produce lipid A, exhibited greatly attenuated endotoxicity but retained adjuvanticity (39, 40). Partial reduction of the

toxicity of lipid A was achieved by removing the C-1 phosphate group to produce MPLA (Figure 3).

The first clinical trial of lipid A in humans has been conducted and the maximum safe dose of intravenous lipid A has been established (41). In other clinical studies, lipid A was incorporated into liposomes with a recombinant malarial antigen and administered to human volunteers (42, 43).

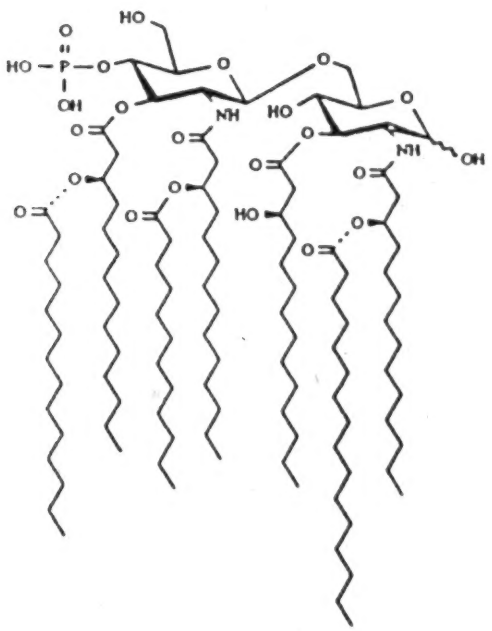

Figure 3. Structure of MPLA.

3. Implications for Vaccine Design

A vaccine should be designed specifically for each particular disease because protective immunity depends on the type of disease.

For example, studies have suggested that type 1 associated immune responses including cellular and/or humoral immunity are protective against tumors and intracellular infections (20, 44, 45). It is possible to direct the immune system toward a desired or protective immunity by using appropriate formulations including adjuvants as mentioned earlier.

The type 1 and type 2 phenotypic distinction can be related to the study of T cell responses in certain intracellular diseases in mice, indicating that it has pathophysiological significance. The most informative studies are those in which mice have been infected with *Leishmania major*. *L. major* is a protozoan parasite that establishes an intracellular infection of macrophages, where it is mainly found in phagolysosomes (46). Protection against *L. major* infection depends upon vigorous macrophage activation; once activated, macrophages can efficiently destroy intracellular *Leishmania* organism, whereas nonactivated macrophages do not kill these organisms.

It has been found that *Leishmaniae* can cause either a healing or nonhealing infection depending on the cytokine profiles and polarization of Th phenotypes in various systems of host and parasite species. $CD4^+$ T cell clones specific for *L. major* antigens prepared from infected healing C57BL/6 mice produce IFN-γ upon *in vitro* activation and fail to produce IL-4 (47, 48). On the other hand, *L. major* infection induces nonprotective immune responses accompanied with markedly elevated serum levels of IgE and *in vivo* biologic activity of IL-4 in the nonhealing BALB/c mice.

Recently, it was found that the increased severity of infection in pregnant C57BL/6 mice was accompanied by reduced IFN-γ and increased IL-4 production by spleen and popliteal lymph node cells stimulated *in vitro* with *L. major* antigens (49). Furthermore, IgG1 was elevated in the serum of pregnant mice as opposed to an increase of IgG2a in infected but non-pregnant controls. These observations support the existence of a bias toward type 2 cytokine expression during pregnancy and suggest that these cytokines effectively down-regulate the course of a normal type 1 associated response against a parasite infection. Therefore, the development of type 1 or type 2 like immune responses can be critical determinants of resistance or susceptibility.

There are a few examples, however, in which IL-4 production by $CD4^+$ T cells has been shown to be protective. One such instance is infection of mice with the nematodes *Heligmosomoides polygyrus*. This parasite has an extracellular habitat. Mice that have been cured of an *H. polygyrus* infection fail to display immunity to rechallenge with the parasite if IL-4 is neutralized (50). Thus, an appropriate vaccine should be designed towards a protective immunity, either in Th1 or Th2 dominance, based on the particular types of diseases.

B. Cancer Vaccines

Vaccines are traditionally used for prophylactic purpose to protect individuals from infection by pathogens such as bacteria and viruses.

However, new technologies for producing vaccine antigens and adjuvants have spurred efforts to develop immunotherapeutic vaccines. One of the most exciting area in immunotherapuetic vaccines is cancer vaccine or active specific immunotherapy (ASI) of cancer (51-54). The concept of ASI is to actively induce immune responses specifically to the antigen by immunization with a well-defined antigen in a proper way. The goal is to stimulate immune attack on established cancers.

1. Immune Surveillance Theory

The concept of developing cancer vaccines was based initially on the premise that there are quantitative and/or qualitative differences between tumor cells and most normal cells. The hypothesis that the immune system might be an effective way to detect these differences and deal with the tumor is also called immune surveillance. There have been four stages on the development of the immune surveillance theory (51). The first stage was called "euphoria" in the late 1950s, when the tumor antigen was first demonstrated by transplantation test. When a tumor was grafted onto a host previously immunized with the inactivated cells from the same tumor, rejection to the graft was observed. This tumor resistance was directed at tumor-associated transplantation antigens (TATAs) of two types. The first are antigens which are shared by many tumors and the second are antigens which are specific to an individual tumor (tumor-specific transplantation antigens, TSTAs). Tumors may express both specific and shared antigens.

The second stage of immune surveillance was called "pessimism" in the late 1960s, when it was realized that many of the very immunogenic TSTA expressed by transplantable tumor cell lines were not natural since they were induced by strong chemical carcinogens, and were not expressed on common spontaneous human cancers (51). With accumulation of knowledge on biology, etiology and antigenicity of human cancers, it was the time of the third stage, "realism." Discovery of a number of tumor antigens has led to the current fourth stage: "optimism."

Tumors are initiated by alterations in the expression of one or several genes that regulate cell growth and differentiation. Such changes can be caused by chemical agents or some viruses. For tumors associated with viral infection, such as hepatocarcinoma and hepatitis B virus, the evaluation of vaccines containing selected virus components is then a logical approach (55). Unfortunately, tumors without an established viral etiology are far more common in humans and this makes the design of suitable vaccines a more difficult task. One of the most promising approaches is to use altered or mutated proteins that are expressed on tumors as the antigen in vaccine formulations. The identification of human tumor rejection antigens opens new possibilities for systematic approaches to the specific immune therapy of cancer (56). Attempts to identify the target antigens by biochemical fractionation of tumor cells up to now have failed, with the important exception of the

identification of underglycosylated mucins present on breast and pancreatic carcinomas.

2. MUC1 Mucin

Mucins are high molecular weight glycoproteins with a very high carbohydrate content (50-90% carbohydrate weight), and are produced by glandular epithelial cells of a variety of tissues, as well by tumors originating from these tissues (adenocarcinomas) (57). Mucin molecules have important functions in protection, lubrication and cell communication. They are expressed as cell surface molecules and as well as secreted molecules by a variety of normal and malignant epithelial cells (58). Pancreatic and breast mucins are both encoded by gene MUC1, which was the first characterized mucin (52, 59, 60). The MUC1 core peptide is composed of a large extracullar N-terminal domain, a transmembrane region, and a cytoplasmic C-terminal domain (Figure 4). The N-terminal domain is mainly made of a central region of tandem repeat sequence of the 20-amino acid sequence PDTRPAPGSTAPPAHGVTSA. The size of the mucin polypeptide core varies from 25 to 100 or more of the tandem repeats. MUC1 is now known to be expressed also on ovary and lung adenocarcinomas, prostate tumors, salivary gland tumors and multiple myelomas (61).

Adenocarcinomas differ from normal epithelial cells with respect to the expression of mucin molecules (51, 61). On normal epithelial cells,

MUC1 expression is polarized and restricted to the apical surface which faces into the lumen of the duct ('outside the body'). It is never found on the basolateral side ('inside the body'), or the sides that border adjacent cells and form very tight junctions (61) (Figure 5).

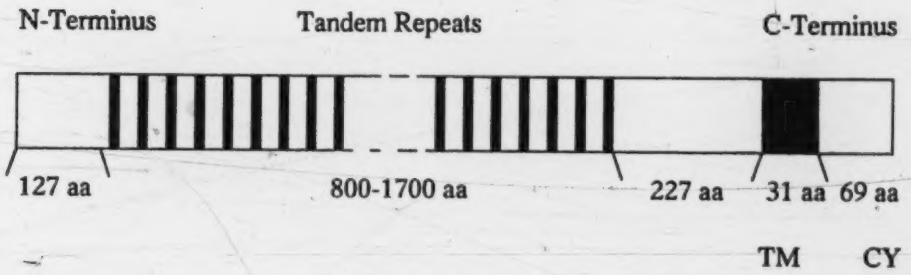

Figure 4. Diagram of MUC1 core peptide, showing a large extracellular domain with a central tandem repeat region flanked by degenerate repeat regions. The C-terminus also contains the transmembrane (TM) and cytoplasmic (CY) regions. (Based on reference 52, 60).

However, when the integrity of the normal duct is compromised by transformation of one of its cells, several very important changes take place which make immune recognition of MUC1 peptide possible. First, the architecture of the duct is destroyed and its lumenal side is more accessible to surveillance by the immune cells. Second, expression of mucin on tumor cells is not polarized anymore, and it covers the entire cell surface (61). Third, the level of expression of mucin on tumor cells is 10-fold higher than on normal cells. Finally, the most important

difference is that mucin molecules produced by adenocarcinomas are underglycosylated (Figure 5). The carbohydrate side chains of malignant cells tend to be fewer and shorter than those of normal cells. This results in the exposure of novel, formerly hidden epitopes on the mucin polypeptide core (61).

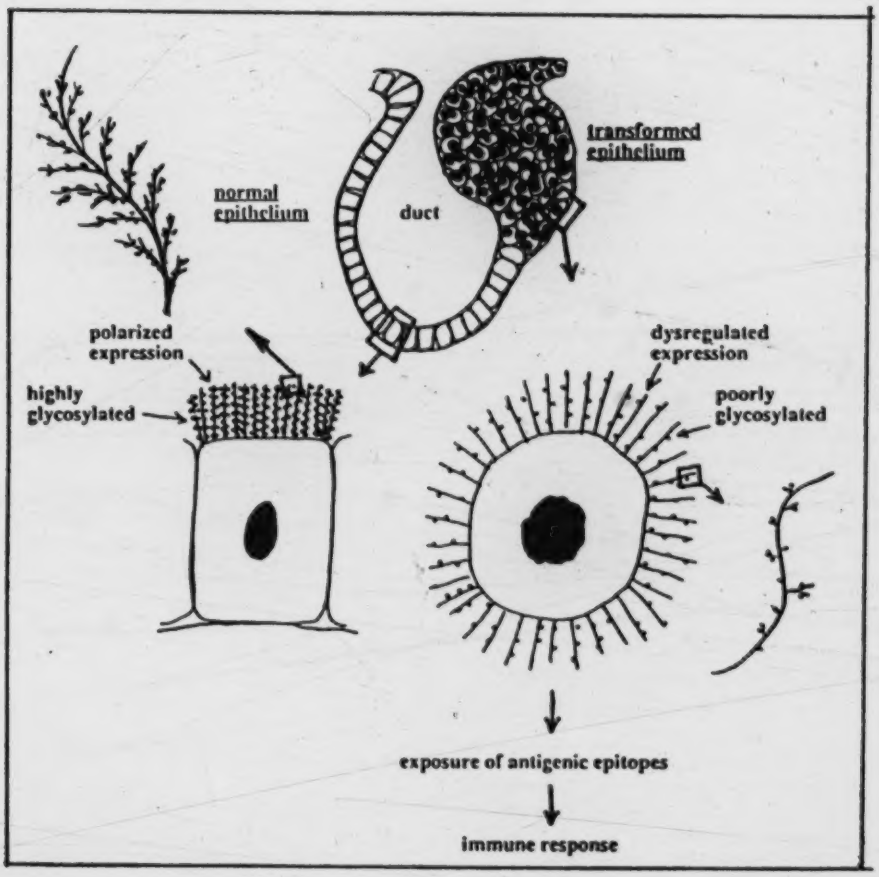

Figure 5. Schematic representation of expression of human mucin MUC1 on normal and transformed epithelium. (Based on reference 61)

3. MUC1-Specific Immune Responses

Normal cells make 'normal mucin' that does not react with known tumor-specific antibodies, nor with tumor specific T cells, presumable because it is fully glycosylated (61). The exposure of certain antigenic epitopes of MUC1 mucins by malignant cells has resulted in its recognition by the immune system (62, 63). The peptide sequences exposed on MUC1 mucin as a result of underglycosylation have been characterized by anti-MUC1 monoclonal antibodies specific for the epitopes of PDTRP sequence of the 20 amino acid unit. These monoclonal antibodies specifically bind to MUC1 mucins on human breast cancer cells, but do not bind to the mucins on normal epithelial cell.

By stimulating draining lymph node cells of pancratic cancer patients with an allogeneic pancreatic tumor cell line, CTLs that lysed most pancreatic tumor cell lines were obtained (64, 65). These were $CD8^+$ CTL with $\alpha\beta$ T cell receptors recognizing cancer associated MUC1 in an antigen specific manner. Normal breast epithelial cells or tumor cells not expressing MUC1 were not lysed by these CTLs.

The hypothesis that underglycosylation in cancer associated mucins seems to unveil epitopes of the repeated peptide to the immune system is furthermore supported by experiments carried out with MUC1 mucin transfected B cells. These cells stimulated the proliferation of anti-mucin CTL clones, but they were not significantly lysed by CTL unless their glycosylation was inhibited (66). Interestingly, these CTLs

functioned independently of MHC molecules (64, 66). The exact mechanism of the apparent MHC-unrestricted antigen recognition by these CTLs is not clear. It was proposed that highly multivalent antigen would bind to multiple T cell receptors increasing the overall avidity of the interaction and trigging T cell function (61). However, anti-MUC1 CTLs induced in mice are different from those obtained from patients. It has been shown that anti-MUC1 CD8+ CTLs are MHC-restricted in mice (67).

Recently, evidence of the protection against breast cancer through natural immunization against MUC1 epitopes during pregnancy suggests that synthetic MUC1 peptide may be used as a cancer vaccine candidate for both prophylactic and therapeutic applications (68-70). By studying MUC1-specific T cell lines, it has been demonstrated that T cells from biparous women tested, but not from nulliparous women or men, have circulating $CD4^+$ T cells which can specifically respond to a MUC1 peptide, presented in the context of autologous APCs (68). Using this experimental model system, it is further demonstrated that MUC1 peptide specific MHC class I restricted CTLs can be generated *in vitro* using T cells from multiparous women stimulated with synthetic MUC1 peptide-loaded, autologous APCs (69). The presence of MUC1-reactive T cells in multiparous women is consistent with their potential role in immune surveillance and provides a rationale for the use of certain synthetic MUC1 peptides for ASI of human carcinomas.

This is further supported by another report describing a long-term survivor of breast cancer whose tumor strongly expressed the T and B cell stimulatory epitopes (70). Five years after presenting with the tumor, the patient had her first pregnancy. It was demonstrated that the lactating breast produced mucin expressing the same tumor-specific epitopes as the original cancer. The patient had circulating anti-mucin antibodies which were not found in normal controls, and mucin-specific CTL in the peripheral blood. The patient remains clinically-free of carcinoma after 5 additional years of follow-up. It was proposed that the original tumor primed the patient's immune response against the mucin epitopes, and that the re-expression of these epitopes on the lactating breast evoked a secondary immune response. It was speculated that the vigor of her anti-mucin immunity might have helped protect this patient against recurrent tumor.

4. Synthetic MUC1 Peptides as Candidates for Cancer Vaccines

Studies described above (61, 68) suggest that MUC1 expressed on tumor cells is an important target antigen for immune responses against breast cancer. These observations provide the basis for using synthetic MUC1 peptide as a vaccine candidate for ASI of adenocarcinomas. As expected, studies of using synthetic MUC1 peptide as the target antigen have shown promising results, however, the protection mechanisms against MUC1-expressing tumors are not clear yet.

Cellular immune responses rather than humoral immune responses have been suggested providing significant anti-tumor effects (71-73). Immune response evaluation of synthetic MUC1 formulations on protection against human MUC1 expressing murine mammary epithelial tumors in mice, Ding *et al* showed that good protection was accompanied with strong and specific MUC1 delayed-type hypersensitivity (DTH) reactions and poor IgG production. However, less effective protection was accompanied with both DTH reactions and humoral immune responses (71). By using a human MUC1 expressing murine tumor model, Apostolopoulos *et al* also showed that the rejection of $MUC1^+$ murine tumor cells by mice was accompanied with generation of $CD8^+$ CTL, $CD4^+$ DTH and little anti-MUC1 antibodies. On the other hand, when mice were immunized with synthetic MUC1 peptides, less tumor protection was observed along with strong antibody responses and a DTH reaction but no $CD8^+$ CTL (72). In another study, however, significant protection against MUC1 expressing tumor cell challenge was observed in mice immunized with MUC1-KLH plus QS-21(74). The protection was accompanied with high levels of MUC1 specific IgG1 and IgG2a antibodies. There was no CD8+ CTL or proliferative T cell response including DTH in those mice.

5. Synthetic Peptide-based Vaccines

An ideal vaccine is expected to mimic the immunogenic epitopes associated with the natural infection, to evoke minimal side-effects and to

be stable and readily available (75). In comparison to conventional vaccines, synthetic peptides are chemically defined and have been postulated to be especially safe. However, the disadvantage of low immunogenicity associated with most of the synthetic peptides has to be overcome. Peptides are known as weak antigens especially for the induction of humoral immune responses. Despite use of protein carriers and adjuvants, many peptides are still reputed for extremely poor antigens. For example, it was reported that an immunization schedule that consisted of sequential subcutaneous (s.c.) immunization with complete Freund's adjuvant, followed by s.c. immunization with incomplete Freund's adjuvant, then followed by intraperitoneal (i.p.) immunization with alum, resulted in only 1 out of 13 peptides derived from hepatitis B surface antigen exhibiting immunogenicity. Of the 13 peptides that were coupled to KLH as a carrier protein, only 7 elicited an anti-peptide humoral immune response (76).

To render peptides immunogenic, they are usually coupled with large carrier proteins containing multiple T cell determinants, such as KLH or TT, and administrated in combination with strong adjuvants (75). However, these approaches have been suggested not proper for human vaccines (77, 78). For example, carrier-induced immune suppression is reported after using TT as a carrier and, in some cases, the strong immunogenicity of the carrier can overwhelm the immune response to the peptide of interest.

The only adjuvant registered and used widely for general medical purpose is still alum, which refers to aluminum compounds including aluminum phosphate, aluminum hydroxide and alum-precipitated vaccines. However, alum is limited to induction of humoral immune responses only (35). The emergence of new generation vaccines such as synthetic peptides has renewed demands for novel adjuvants that are acceptable for human use as well as highly effective in potentiating both humoral and cellular immunities.

C. Liposomal Adjuvanticity

It has been more than 20 years since the first demonstration of liposomal adjuvanticity of entrapped antigens. In 1974, Allison and Gregoriadis showed that antibody responses were increased up to 10-fold when immunized with liposome-entrapped diphtheria and tetanus toxoids compared to free antigens (79). Since then, the immunoadjuvant properties of liposomes have been repeatedly demonstrated for both humoral and cell-mediated immunity to a wide spectrum of bacteria, protozoan and viral antigens (80, 81).

1. Liposomes as Antigen Delivery System

There are a number of important advantages by using liposomes as immunoadjuvant of antigens. They are biocompatible and biodegradable, immunologically inert, structurally versatile, nontoxic, and able to incorporate a wide range of antigens (82). The availability of

liposomes with variable characteristics in terms of vesicle size, composition, surface charge and mode of antigen accommodation (e.g. entrapped within the aqueous phase or exposed on the liposome surface), suggests versatility in immunoadjuvant action and vaccine design.

A variety of protein and peptide antigens can be incorporated into liposomes (83). Depending on the hydrophobicity of the antigen, it could either be encapsulated in the aqueous phase of liposomes or embedded in the lipid bilayers or partially inserted in the bilayer and partially exposed on the surface of the bilayer. For non-bilayer associating water soluble drugs, encapsulation efficiency depends fully on the compartments in liposomes (84, 85). Therefore, factors influencing the space between lipid bilayers may affect the encapsulation efficiency of the drug.

Besides all the advantages mentioned above, liposomes also can reduce the toxicity of associated molecules. Incorporation of lipid A into liposomes reduces its pyrogenicity greatly (86). The highest dose of lipid A administered to human, a 2200 μg dose, represents a 12-fold increase in the previously reported maximal safe dose of free lipid A (41). Liposomes containing lipid A have shown considerable promise for human vaccines. Recently, clinical trials have shown that utilization of liposomes as the delivery vehicle is an extremely safe and highly potent vaccine formulation for use in humans (87, 88). Liposomes containing

lipid A have been utilized in a Phase I clinical trial of a proposed malaria vaccine. This liposomal malaria vaccine resulted in very high levels of antibodies against the malarial antigen, and the liposomal lipid A was nonpyrogenic and safe for use in humans. In addition, the first commercialization of a human liposomal vaccine (Epaxal Berna®) has recently been accomplished, a vaccine against hepatitis A that has been licensed in Switzerland (89).

2. Liposomal Antigen Presentation

The rationale for using liposomes to carry antigens for immunization was originally based on the known preferential uptake by APCs such as macrophages of parenterally injected liposomes (90, 91). Macrophages were initially believed the major component of APCs in the induction of immune responses due to their apparently strong phagocytic capacity. However, recently, the dendritic cells are found the most potent APCs able to efficiently induce primary T cell-mediated responses to foreign antigens (92, 93). Dendritic cells are cells specialized for antigen capture, migration and T cell stimulation. After capturing antigen in the periphery, they migrate to lymphoid organs where they present the antigen to T cells. Their seemingly unique ability to interact with and sensitize naive T cells gives dendritic cells a central role in the initiation of immune responses and allows them to be used in therapeutic strategies against cancer, viral infection and other diseases. Studies

indicate that these cells participate in antitumor immunity and tumor surveillance (94).

Mechanisms of liposomal antigen presentation have been demonstrated under the assumption that macrophages would serve as the primary APCs for liposomal presentation (95, 96). A model *in vitro* system comprising bone marrow-derived macrophages was constructed to study the intracellular fate of liposomal antigens to T cells (91). Liposomes containing a malaria protein antigen were phagocytosed by macrophages. The appearance of the antigen on the surface of the macrophages was observed within 15 min, after initial incubation of liposomes with the cells, and continuous expression of the antigen on the macrophage surface occurred for a prolonged period (at least 24 h). Subsequent studies have recently confirmed the ability of liposomes to deliver another antigen to the class II pathway of macrophages for MHC restricted presentation to antigen specific T cells (97).

After ingestion by macrophages, liposomes were accumulated along peripheries of the vacuoles, presumably endosomes, as visualized by immunogold electron microscopy, in which a monoclonal antibody specific to the liposomal antigen was used as a probe for detecting the antigen (91). Surprisingly, it also showed that liposomal antigen was subsequently released from the endosomal vacuoles and appeared in large amounts in the cytoplasm of the macrophages. The observation of delivery of liposomal antigen to the cytoplasm of macrophages with non-

pH-sensitive liposomes has also been confirmed with a different antigen (98). Although the exact mechanism of release of liposomal antigen into the cytoplasm is not fully understood, CTL can be induced via the exogenous pathway by liposomal delivery of certain antigens (99).

3. Effect of Formulation Parameters on Liposomal adjuvanticity

To circumvent the difficulties inherent in protein carriers as discussed earlier, two alternative strategies have been proposed to enhance peptide immunogenicity, namely directly conjugated peptide to liposomes followed by emulsification in Freund's adjuvant (100), or in the absence of Freund's adjuvant (101). Although liposomes containing acylated peptides failed to induce lymphoproliferative responses (except by the use of complete Freund's adjuvant), the incorporation of further adjuvants into liposomes, such as lipid A and muramyl tripeptide-phosphatidylethanolamine (MTP-PE), did result in such responses and subsequently strong humoral immune responses (101). Strategies of using acylated peptide in liposomes pointed to the necessity of using an adjuvant in liposomes, however, the role of peptide location in immunopotentiation has not been elucidated.

Antigens can be associated with liposomes in two ways. The antigenic determinant is either masked (encapsulated within the internal aqueous spaces or totally embedded within the lipid bilayers of liposomes) or exposed (covalently-attached or non-specifically adsorbed to the lipid bilayer of liposomes). The relevance of topographical location

of antigen in liposomes on liposomal adjuvanticity has been controversial. Some have reported that the immunogenicity of antigen was not improved after being encapsulated in liposomes (102, 103), but this hypothesis has not been supported by several studies (104, 105). Subsequent studies have shown that humoral responses may be induced by various types of liposomal formulations and the level of antibody production by surface-exposed protein antigens may be higher, lower or similar compared to encapsulated protein antigens (106-109). Besides humoral immune responses, it has been suggested that the topographical location of antigen in liposomes may also affect the type of T cell responses (110).

Expression of peptide-MHC complexes in a sufficient amount at the cell surfaces is necessary to stimulate strong T cell responses (4, 111). Factors influencing such an expression may affect the magnitude of T cell responses. Although there are reports suggesting that lipid composition and liposome size may influence the magnitude of T cell responses, until recently, there has been little information on T cell responses as a function of liposome composition and size in mice (112-114).

D. Aim, Objectives and Hypothesis

1. Aim

Th cells are the central regulating components of the immune system. Th1-associated immune responses have been suggested to provide protection against tumors and intracellular infections. This study

was aimed at developing a liposomal delivery system of synthetic MUC1 peptide antigens favoring type 1 immune responses.

2. Hypothesis

The incorporation of MUC1 peptide in liposomes increases T cell and humoral immune responses in a specific manner depending on the location of the antigen in the liposomes. Liposomes containing MPLA favor the promotion of type 1 associated immune responses.

3. Objectives

1. To formulate and characterize MUC1 peptide liposomes with respect to peptide encapsulation efficiency and retention,
2. To investigate the nature of peptide association with liposomes on the type and magnitude of T cell and humoral immune responses, respectively,
3. To evaluate the roles of liposome composition and size in the magnitude of T cell responses in a homologous mouse model.

II. MATERIALS AND METHODS

A. Materials

Solvents for high performance liquid chromatography (HPLC), including ddH_2O (de-ionized distilled water, Millipore Corporation, Bedford, MA), acetonitrile and methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ) were freshly filtered through 0.22 μm filters (Millipore Corporation, Bedford, MA) prior to use. The Micro BCA (bicinchoninic acid) protein assay reagent kit was purchased from Pierce, Rockford, IL., and ethanol absolute (purity above 98%) from Fisher Scientific. Trifluoroacetic acid, Triton X-100 and phenol were purchased from Sigma Chemical Company, Mississauga, ON. Canada.

Dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC), cholesterol (CHOL), dimyristoyl phosphatidylglycerol (DMPG) and stearylamine (SA) were purchased from Princeton Lipids (Princeton, NJ). All chemicals used for preparing buffers were reagent grade. Monopotassium phosphate, disodium phosphate, sodium chloride, sodium acetate, acetic acid, triethylamine (TEA) and chloroform were purchased from BDH Corporation, Toronto, ON. Canada. Trizma base {Tris(hydroxymethyl)aminomethane}, Trizma hydrochloride {Tris(hydroxymethyl)aminomethane hydrochloride} and polycarbonate

membranes (Nuclepore Corporation, Pleasanton, CA) were purchased from Sigma Chemical Company, Mississauga, ON., Canada.

Mouse MUC1 peptide SP1-020, ovalbumin peptide SP1-037 and human MUC1 peptides BP16, BP24, BP25, BP30 and SP1-023 were constructed using the solid-phase approach of Merrifield (115) on an automated peptide synthesizer (MilliGen/Biosearch Model 9050) (Table I). BLP25 was synthesized using lauryl cysteine as the N-terminal amino acid. Lauryl cysteine was pre-synthesized in solution phase by coupling lauric acid to the amino group of cysteine. BLP24 was synthesized using palmitic lysine glycine as the C-terminal amino acid. All of the above synthetic peptides and monoclonal antibodies (mAb) B248.7R2 (specific for MUC1 peptide sequence APPAHGVTSA) , B80.3 (specific for PSA, prostate specific antigen), R46.A2 (coating mAb in the IFN-γ assay) , XMG1.2 biotinylated (second mAb in the IFN-γ assay) and 11B11 (coating mAb in the IL-4 assay) were produced and provided by Biomira Incorporation, Edmonton, AB., Canada.

MPLA (Ribi Immunochem Incorporation, Hamilton, MT), FITC-conjugated goat anti-mouse IgG1 monoclonal antibody (Southern Biotechnology Associates Incorporation, Birmingham, AL), DETOX adjuvant (Ribi Immunochem Incorporation, Hamilton, MT), mitomycin C (Sigma Chemical Company, Mississauga, ON., Canada), 96-well flat bottom Costar microtitre plates (Fisher Scientific, Fair Lawn, NJ),

Table I. Synthetic Peptides

Synthetic Peptide	Amino Acid Sequence
BP16	GVTSAPDTRPAPGSTA
BP24	TAPPAHGVTSAPDTRPAPGSTAPP
BLP24	TAPPAHGVTSAPDTRPAPGSTAPP-Lys(Palmitic)G
BP25	STAPPAHGVTSAPDTRPAPGSTAPP
BLP25	(Lauryl)Cys-STAPPAHGVTSAPDTRPAPGSTAPP
BP30	RPAPGSTAPPAHGVTSAPDTRPAPGSTAPP
SP1-020	DSTSSPVHSGTSSPATSAPEDSTS
SP1-023	PDTRPAPGSTAPPAHGVTSA
SP1-037	ISQAVHAAHAEINEAGR

^{3}H-thymidine (Amersham Canada Limited, Oakville, ON., Canada), scintillation fluid (Cytoscint Research Production Division, Costa Mesa, CA), BVD6-24G2 biotinylated (second mAb for IL-4 assay, PharMingen, Mississauga, ON. Canada), rIFN-γ and rIL-4 (recombinant IFN-γ and recombinant IL-4, PharMingen, San Diego, CA), GIBCO RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, 2-mercaptoethanol, gentamicin, penicillin and streptomycin (Life Technologies Incorporation, Grand Island, NY) and C57BL/6 or BALB/c or CAF1 mice (Charles River, St-Constant, QC, Canada) were provided by Biomira Incorporation, Edmonton, AB., Canada.

Nunc-immunoplates maxisorp (96-well GIBCO BRL, Life Technologies Incorporation, Grand Island, NY), serum separator tube (Microtainer, Becton Dickinson and Company, Mississauga, ON. Canada), Tween 20 (Sigma Chemical Company, Mississauga, ON., Canada), metofane (Janssen Pharmaceutica, North York, ON., Canada), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratory Incorporation, Bar Harbor, ME), peroxidase labeled goat anti-mouse IgG and IgM antibodies, bovine serum albumin (BSA) and TMB or ABTS peroxidase substrates (Kirkegaard and Perry Laboratories, Gaithersburg, MD), horseradish peroxidase labeled goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies (Southern Biotechnology Associates Incorporation, Birmingham, AL), and FITC-conjugated rat anti-mouse IgG1 mAb (PharMingen, Mississauga, ON. Canada) were purchased from suppliers as indicated.

B. MUC1 Peptide Quantification

It was important to develop a reliable and reproducible assay for the quantification of MUC1 peptides. BCA and HPLC assays were compared for the analysis of MUC1 peptides in buffered solutions and in liposomes.

1. BCA Assay

The BCA protein assay procedure was as described by Smith *et al* (116). The standard working reagent was prepared fresh for each assay

by mixing 50 parts of reagent A (1% disodium bicinchoninate in sodium carbonate, sodium bicarbonate, sodium tartrate and sodium hydroxide), 48 parts of reagent B (aqueous solution of BCA detection reagent) and 2 parts of reagent C (4% $CuSO_4 \cdot 5H_2O$).

In a standard assay, a certain volume of BP30 stock solution in saline (0.9 g NaCl /100 ml de-ionized H_2O) containing 1 μg to 50 μg or 10 μg to 100 μg of the peptide was mixed with an equal volume of freshly prepared working reagent. The mixture was incubated in a 60°C water bath for 60 min. Once the samples had cooled to room temperature, the absorbances were measured at 540 nm on a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) with a reagent saline mixture as the blank reference.

To study if the lipids could influence the absorption of the peptide, a certain amount of empty liposomes (DMPC:CHOL:DMPG, 3:1:0.25, mole ratio) was added to each of the peptide preparations, followed by mixing with the working reagent and incubation. The lipid to peptide ratio (w / w) was 20:1.

2. HPLC Analysis

HPLC assays were developed and optimized for MUC1 peptides.

a. In solution

MUC1 peptides in buffered solution or the supernatant of a liposome formulation was mixed with phenol that served the internal reference. A volume of 50 μl from each of the prepared samples was

manually injected to the HPLC system for each analysis. HPLC conditions for each of the MUC1 peptides had been optimized to obtain a smooth and straight baseline, with proper separation of the eluting peaks of the peptide and the internal reference phenol. Each analysis lasted about 25 min.

A Waters 625-746 HPLC system with a C-18 reverse-phase radial-pak cartridge (8 mm x 100 mm) was employed for the analysis of MUC1 peptides. A standard procedure was followed for the operation of the HPLC system. A specifically programmed gradient aqueous mobile phase for each of the MUC1 peptides consisting of eluent A: 10% acetonitrile with 0.1% trifluoroacetic acid (TFA) and eluent B: 70% acetonitrile with 0.085% TFA was used. Peptides were detected by a UV detector at absorbance of 210 nm and the flow rate was 1 ml / min. Quantification of a MUC1 peptide was done by using a calibration curve based on the ratio of peak areas of the peptide and phenol.

b. in liposomes

To develop an analysis of MUC1 peptides encapsulated in liposomes, a suitable dissolving agent of liposomal pellets was determined. Various amounts of Triton X-100 (1%-5%) or ethanol (10%-70%) were added to a solution containing a known quantity of peptide and/or phenol and possible interferences were determined.

Peptide recovery was determined using empty liposomes spiked with a known amount of peptide. After centrifugation of empty liposomes,

the pellets were dissolved in various volumes of ethanol. Known amounts of peptide and phenol were added, followed by HPLC injection. The optimal amount of ethanol for liposomes of various phospholipid compositions was also determined. Mass balance measurements were made to confirm peptide recoveries after incubation aliquots of liposomes at 37^0C for 24 h or at 4^0C for a month. At various time intervals, liposomes were centrifuged and both pellet and supernatant were analyzed for peptide by HPLC as described above.

C. Liposome Formulation

1. Physical Characterization as a Function of:

a. charge and composition

Solutions of lipids (DMPC, DPPC or DSPC and CHOL in chloroform, DMPG or SA in chloroform:methanol, 3:1, v/v) at 3:1:0.25 mole ratios of PC:CHOL:PG or SA were mixed in a round-bottom flask. Using a rotary evaporator (Lab-Line Instruments Incorporation, Melrose Park, IL) (200 rpm and 40°C), the organic solvent was removed under negative pressure with the aid of a cold water condensation system and a dry-ice acetone (wash) bath. The flask with lipid film was further dried in a vacuum desiccator (Model 524, Precision Scientific Corporation, Chicago, IL) over night at 40°C.

Hydration of the dried lipid film was carried out in the rotary evaporator (60 rpm) at 12°C above the phase transition temperature (Tc) of the specific phospholipid used. Specifically, the temperature used in lipid hydration was 35°C, 53°C and 65°C for DMPC, DPPC and DSPC liposomes, respectively. The contents of the flask containing the newly-formed multilamellar vesicles (MLVs) were vortex-mixed (Maxi Mix II-Thermolyne). Care was taken to disperse all of the dried lipid from the walls of the flask.

MLVs containing MUC1 peptides BP24, BP25 or SP1-020 at a final lipid concentration of 30 mM were prepared by the freeze-and-thaw (FAT) method. Freezing was carried out in a dry-ice acetone bath for 5 min, then the preparation was allowed to thaw at room temperature for 50 min. After thawing, the flask was gently swirled in a water bath for 5 min at a temperature which was the same as that used in dispersing the lipid, followed by gentle vortex-mixing. The above process was repeated five times in all cases.

After centrifugation of the freshly-formed FAT MLVs at 150,000 x *g* for 12 min (Ultracentrifuge Model L8-55, Beckman Instruments, Palo-Alto, CA), the supernatant was removed and saved for HPLC analysis. The pellet of FAT MLVs was washed once under the same conditions with buffered solution. Supernatants from washings were collected for peptide analysis, followed finally by resuspension of pellets in buffered solution and stored at 4°C. Care was taken to measure the volumes of liposome

supernatants and of the buffered solution used for re-suspending the pellets, in order to maintain a total lipid concentration of 30 mM. Encapsulation efficiency was expressed as the ratio of the amount of peptide found in the liposomal pellet to the amount of peptide found in both pellet and supernatant.

b. pH and ionic strength

After drying the lipid film, an appropriate hydration solution of the desired pH and ionic strength containing MUC1 peptide BP24 or SP1-020 was added. Buffered solutions of different pH were prepared as follows: acetate buffer (sodium acetate and acetic acid) was adjusted to pH 4 or 5.5 by 1M sodium hydroxide; phosphate buffer solution (PBS, monopotassium phosphate, disodium phosphate and sodium chloride) and Tris buffer (Trizma base and Trizma hydrochloride) were adjusted to pH 7.4 by 1M sodium hydroxide. The ionic strength of the buffered solution was adjusted with sodium chloride.

2. Immunization

a. different doses of BP24

Solutions of lipids with predetermined mole ratios (DPPC or DSPC:CHOL:DMPG, 3:1:0.25) were mixed in a round-bottom flask. A known amount of immunomodulator, MPLA (10 mg per vial), was dissolved in a specific volume of chloroform:methanol, 4:1, v/v solution, then measured volumes were diluted in separate vials to obtain a range of concentrations. An aliquot of MPLA solution corresponding to 1% w/w

of the total lipids was then added to the round-bottom flask and mixed with the lipid solutions, followed by drying and desiccating over night.

After drying, an appropriate hydration solution (filter-sterilized PBS, pH 7.4 and ionic strength 0.15 M or 0.04 M) containing various amounts of BP24 (see below) was added to each of the round-bottom flasks. The desiccated lipid films were dispersed at 53°C or 65°C followed by five cycles of FAT, swirl-incubation and vortexing.

The hydration concentration of BP24 was calculated based on the desired peptide injection dosage and the encapsulation efficiency of the peptide. The actual BP24 dosages in liposomes used for injection in mice were quantified by HPLC analyses of both the supernatant and liposomal pellets. After centrifugation, supernatants containing untrapped peptides were saved for HPLC analysis. Freshly-made FAT MLVs containing various amounts of BP24 was removed for determination of peptide in the liposome pellet by HPLC.

Two sets of liposomes with a range of BP24 peptide dosages were formulated. One set was 100 ng, 500 ng, 5 μg or 16 μg BP24 peptide per 100 μl of the liposomes, and the other set was 7 μg, 24 μg or 81 μg per 50 μl. Samples for flow cytometric analyses ('liposomal BP24' and 'liposomal BLP24') were formulated at a peptide dose of 6 nmole per 50 μl liposomes.

b. formulations of BP25 and BLP25

Each of the formulations was mixed with 1% w/w MPLA at fixed concentrations of peptide (2 nmole per 50 µl injection sample: 5 µg BP25 or 5.6 µg BLP25). Empty liposomes containing 1% w/w MPLA were prepared as controls. A solution with pre-determined mole ratios of lipids (DSPC:CHOL:DMPG, 3:1:0.25) and MPLA were mixed together in round-bottom flasks. After drying, an appropriate hydration solution (filter-sterilized PBS, pH 7.4 and ionic strength 0.04 M) containing BP25 or no peptide was added to each of the round-bottom flasks. The hydration concentration of BP25 was calculated based on the peptide injection dose and the encapsulation efficiency of the peptide. The desiccated lipid films were dispersed at 65°C, followed by five cycles of FAT, swirl-incubation and vortex-mixing. The resulting FAT MLVs were 'liposomal BP25' or 'empty' (peptide free) liposomes.

Lipopeptide BLP25 in chloroform:methanol, 4:1, v/v solution was mixed with the solution of lipids (DSPC:CHOL:DMPG, 3:1:0.25) containing 1% w / w MPLA prior to drying. After desiccation the lipid film was dispersed in an appropriate volume of filter-sterilized PBS without peptide at pH 7.4 and 0.04 M ionic strength. This formulation was named 'liposomal BLP25'. Freshly-made 'liposomal BP25' or 'liposomal BLP25' (100 µl) was analyzed for peptide by HPLC. After centrifugation, supernatants of 'liposomal BP25' or 'liposomal BLP25' were collected for

HPLC analyses. The liposomal pellets were re-suspended in sterilized PBS and stored at 4°C for immunization of mice.

A specified volume of BP25 stock solution of a known peptide concentration was mixed with 'empty' liposomes by gentle vortex-mixing. This yielded 'BP25 mixed' liposomes at a dose of 5 μg BP25 per 50 μl of each injection. A control lipopeptide BLP25 formulation was prepared based on the solubilization instructions for MPLA (Ribi Immunochem Incorporation, Hamilton, MT). MPLA (400 μg) in chloroform and methanol was dried at 40°C for 20 min in a 100 ml round-bottom flask by rotary evaporation under negative pressure. An aqueous solution of 0.1% v/v TEA in ddH_2O was added to the flask containing dried MPLA. The mixture was swirled at 65°C for 30 sec followed by sonication for 30 sec. The incubation and sonication procedures were repeated until the solution was clear. A specified volume of BLP25 solution was added and mixed with the MPLA solution to produce a formulation with 5.6 μg BLP25 per 50 μl. This preparation was called 'BLP25 alone'.

c. SP1-020 DETOX adjuvant preparation

A specified amount of SP1-020 (see below) was added to a certain amount of the DETOX adjuvant (an o/w emulsion from Ribi Immunochem Incorporation, Hamilton, MT) and vortex-mixed for 10 min before injection into mice. The SP1-020 peptide dose was 5 μg, 50 μg or 250 μg per 50 μl of the emulsion.

d. as a function of SP1-020 dose and phospholipid composition

Lipid solutions with pre-determined mole ratios (DMPC, DPPC or DSPC:CHOL:DMPG, 3:1:0.25) were mixed with a MPLA solution (1% w/w) in round-bottom flasks. The desiccated lipid films were dispersed in filter-sterilized PBS containing SP1-020 as described earlier followed by five cycles of FAT. The hydration concentration of SP1-020 was calculated based on the desired peptide injection dose and the encapsulation efficiency of the peptide at the particular phospholipid composition. After HPLC analyses, the SP1-020 peptide dosage of per 50 μl liposome was found to be 4.5 μg and 92 μg for the immunization in CAF1 mice; 5 μg and 35 μg for the immunization in C57BL/6 mice; 12 μg, 21 μg, 30 μg, 60 μg and 100 μg for the immunization in BALB/c mice. A SP1-020 peptide dosage of 32 ± 2 μg per 50 μl liposome suspensions was obtained.

e. as a function of liposome size

Various sizes of liposomes containing the mouse MUC1 peptide SP1-020 were generated by the following two methods after SP1-020 was encapsulated into FAT MLVs as described above.

(i). sonication method

SP1-020 FAT-MLVs (DSPC:CHOL:DMPG, 3:1:0.25; 1% w / w MPLA) varying in sizes were obtained by a microprobe sonication method (Heat Systems Incorporation, Farmingdale, NY). The probe was immersed in the liposome suspension approximately 4 mm below the

surface. Liposomes of certain volumes were sonicated for a certain period of time at power level 4. The sizes of the resultant liposome samples were measured as described below.

The sonication process was carried out in an ice-bath and repeated until a range of expected sizes of liposomes was obtained. Then the liposome suspensions were centrifuged (Model 5415C Eppendorf Centrifuge, Brinkmann Instruments Canada Limited, Mississauga, ON. Canada) to remove oversized lipid aggregates and metal particles from the titanium probe of the sonicator. The various sizes of liposomes (100 μl) was taken from each sample for peptide HPLC quantification.

(ii). membrane extrusion method

Mouse MUC1 peptide SP1-020 was incorporated in FAT-MLV liposomes of DSPC:CHOL:DMPG, 3:1:0.25 (mole ratio) and 1% (w / w) MPLA as described above. Liposomes of various sizes were obtained by extrusion (Lipex Biomembranes Extruder, Vancouver, BC) through polycarbonate membranes (pore sizes of 100, 200, 400, 600 or 800 nm). This extrusion process was repeated until a range of the expected sizes of liposomes were generated as measured by dynamic light scattering (see below). The various sizes of liposomes (100 μl) was taken from each sample for peptide HPLC quantification.

3. Size Analysis

Liposome size and size distribution were determined by dynamic light scattering (Brookhaven Instruments Corporation, Holtsville, NY).

Liposome suspensions were diluted 300 times with the buffer used in formulation, then the samples were allowed to equilibrate for 15 min at room temperature followed by the size measurement at 25 °C.

D. Peptide Retention at 37°C and 4°C

1. In PBS

Aliquots of liposomes encapsulated BP24 (DMPC, DPPC or DSPC:CHOL:DMPG, 3:1:0.25, mole ratio) or BP25 and BLP25 (DSPC:CHOL:DMPG, 3:1:0.25, mole ratio) were incubated in a shaking water-bath (Dubnoff Metabolic Shaking Incubator, Precision Scientific Corporation, Chicago, IL) at 37°C for 24 h, or stored in 4°C refrigerator from 31 days to over one year. At various time intervals, liposome samples were centrifuged and analyzed by HPLC for MUC1 peptide presence in the supernatant and the liposomal pellet.

2. In serum

Blood was collected via cardiac puncture in serum separator tubes (Microtainer) under sterile conditions, after C57BL/6 mice were anesthetized with metofane. Serum was obtained after centrifuging the blood containing serum separator tubes for 4 min (Model 5415C Eppendorf Centrifuge, Brinkmann Instruments Canada Limited, Mississauga, ON. Canada). Human serum of healthy human volunteers was obtained from the Red Cross, University of Alberta Hospital, Edmonton, AB., Canada. Pooled sera from mice or humans were used

for each set of the retention experiments. Sera were handled strictly according to the biosafety regulations of Biosafety Office (University of Alberta) in a laboratory with a Human Body Substance Permit.

Aliquots of liposomes encapsulated SP1-020 (DMPC, DPPC or DSPC:CHOL:DMPG, 3:1:0.25, mole ratio), BP25 or BLP25 (DSPC:CHOL:DMPG, 3:1:0.25, mole ratio) were centrifuged and the supernatants were replaced with the same volume of pure mouse serum for SP1-020 liposomes or human serum for BP25 or BLP25 liposomes. After mixing, samples were incubated in a shaking water-bath (Dubnoff Metabolic Shaking Incubator, Precision Scientific Corporation, Chicago, IL) at 37°C for 24 h. At various time intervals, liposome samples were washed twice with physiological PBS and the liposomal pellets were analyzed for MUC1 peptides by HPLC. During HPLC analyses of BLP25 liposomes, eluted BLP25 peptides were collected for mass spectrometric analysis. In addition, the incubated liposome samples was taken for size determination at various time points.

E. Flow Cytometric Analysis

To determine the surface exposure of a human MUC1 peptide epitope, aliquots of 50 μl of liposomes, including peptide free 'empty liposomes', BP25 mixed with 'empty liposomes', BP25 or BLP25 entrapped liposomes ('liposomal BP25' and 'liposomal BLP25'), BP24 or BLP24 encapsulated liposomes ('liposomal BP24' and 'liposomal BLP24')

were incubated with B248.7R2 (MUC1 specific mAb for the amino acid sequence APPAHGVTSA of the human MUC1 peptide) or B80.3 (control mAb, specific for PSA: prostate specific antigen) for 45 min at 4 °C. After washing by centrifugation (1-2 min at 14,000 rpm, Eppendorf Centrifuge) with 1% bovine serum albumin (BSA) containing PBS (pH 7.4, ionic strength 0.15 M), PBS was added to each sample and vortex-mixed, then fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG1 antibodies was added to each sample tube, followed by incubation at 4 °C for 30 min. The liposome samples were then washed twice with 1% BSA containing PBS and PBS, respectively. After washing by centrifugation, the samples were vortex-mixed with 400 µl of PBS and analyzed using a fluorescence activated cell sorter (FACS) flow cytometer (Becton Dickinson, Mountain View, CA) under standard conditions.

F. Mass Spectrometric Analysis

BLP25 peptides in acetonitrile and water collected during HPLC pellet analyses of 100% human serum incubated liposomes were analyzed by a VG Trio 2000 electrospray mass spectrometry system (Micromass Ltd., Manchester, England) under a solvent system of acetonitrile and water (50:50, v/v) at 79°C and 46 voltage. This mass analysis was performed by Jane Nagel.

G. Immunization Procedures

1. Human MUC1 Peptide Formulations

a. BP25 and BLP25 preparations

(i). in C57BL/6 mice

Normal serum samples were collected from C57BL/6 mice (female, 7-8 week old) by tail vein bleeding one day before immunization.

Single Immunization:

On day 0, five groups of mice (seven in each group) were immunized with each of the following five formulations: 'empty', 'BP25 mixed' with empty liposomes, 'BLP25 alone', 'liposomal BP25' and 'liposomal BLP25'. Each of the peptide preparations (50 μl) was injected subcutanously (s.c.) in the left hind footpad of each mouse.

On day 10, blood was collected from each mouse by cardiac puncture and the draining popliteal lymph nodes of the immunized mice were taken for T cell proliferation assay. Spleens of unprimed C57BL/6 mice were also collected for preparation of the APCs.

Two Immunizations:

On Day 0, another five groups of mice (seven mice in each group) were immunized with each of the same five BP25 and BLP25 formulations. Each of the peptide preparations (50 μl) was injected s.c. in the left thigh of each mouse.

On day 14, an identical second immunization was given intraperitoneally (i.p.) to each mouse.

On day 26, blood and the draining inguinal lymph nodes of the immunized mice were collected. Spleens of unprimed C57BL/6 mice were also taken for the T cell proliferation assay.

(ii). in BALB/c mice

Normal serum samples were collected from BALB/c mice (female, 7-8 week old) by tail vein bleeding one day before the immunization.

On Day 0, five groups of mice (three in each group) were immunized with each of the five following formulations: 'empty', 'BP25 mixed' with empty liposomes, 'BLP25 alone', 'liposomal BP25' and 'liposomal BLP25'. Each of the peptide preparations (50 μl) was injected s.c. in the left thigh of each mouse. On day 14, an identical second immunization was given i.p. to each corresponding mouse. On day 26, blood was collected by cardiac puncture from each of the immunized mice.

b. BP24 liposomes

On day 0, three groups of BALB/c mice (female, 7-8 week old, four mice in each group) were immunized with BP24-encapsulated liposomes with a peptide dosage of 7 μg, 24 μg or 81 μg in per 50 μl of the liposome samples. Each of the peptide preparations (50 μl) was injected s.c. in the left hind footpad of each mouse.

On day 10, the draining popliteal lymph nodes of the immunized mice and spleens of unprimed BALB/c mice were collected for the T cell proliferation assay.

2. Mouse MUC1 Peptide Formulations

a. liposomes

(i). in CAF1 and C57BL/6 mice

On day 0, groups of CAF1 or C57BL/6 mice (female, 7-8 week old, ten or five in each group) were immunized s.c. in the left hind footpad with saline alone or SP1-020 liposomes of a peptide dosage of 4.5 μg or 92 μg in CAF1 mice and 5 μg or 35 μg in C57BL/6 mice per 50 μl of the liposome samples.

On day 10, the draining popliteal lymph nodes of the immunized mice and spleens of unprimed CAF1 or C57BL/6 mice were collected for the T cell proliferation assay.

(ii). in BALB/c mice

On day 0, groups of BALB/c mice (female, 7-8 week old, five in each group) were immunized with liposomes of various amounts of SP1-020 (12 μg, 21 μg, 30 μg, 60 μg or 100 μg per 50 μl) or various phospholipid compositions (DMPC, DPPC or DSPC:CHOL:DMPG, 3:1:0.25, mole ratio) or various sizes generated either by the sonication method (123 nm, 217 nm, 285 nm, 410 nm, 614 nm) or by the membrane extrusion method (146 nm, 218 nm, 305 nm, 641 nm, 905 nm). Each of the peptide preparations (50 μl) was injected s.c. in the left hind footpad or in both thighs of each mouse (only in the case of sizes obtained by the membrane extrusion method). On day 10, the draining popliteal or

inguinal lymph nodes of the immunized mice and spleens of unprimed BALB/c mice were collected for T cell proliferation assay.

b. SP1-020 DETOX emulsions

On day 0, three groups of BALB/c mice (female, 7-8 week old, four in each group) were immunized with SP1-020 DETOX at peptide dosages 5 μg, 50 μg or 250 μg per 50 μl of the o/w emulsion samples. Each of the peptide preparations (50 μl) was injected s.c. in the left hind footpad of each mouse. On day 10, the draining popliteal lymph nodes of the immunized mice and spleens of unprimed BALB/c mice were collected for T cell proliferation assay.

H. Evaluation of Immune Responses

1. T Cell Proliferation Assay and Cytokine Collection

Chemically inactivated syngenic spleen cells from naïve mice were used as APCs. Mitomycin C was added at a dose of 60 μg per 20 million spleen cells and incubated for 100 min at 37°C in 7% CO_2 followed by washing (5 times) with 5% fetal bovine serum (FBS) containing RPMI-1640 medium.

T cells (recovery rate of 22-30%) were isolated from the lymph node cells by the nylon wool purification method (117). T cells of various concentrations (2.5×10^5 / well or 5×10^5 / well) and APCs (5×10^5 / well) were cultured in a total volume of 300 μl in 96-well flat bottom Costar microtitre plates in the presence of media alone (peptide-free

background), or the recall peptide antigens of SP1-020, BP24 or BP25 at a concentration of 20 μg / well for a period of 24 h, 48 h, 72 h or 96 h at 37°C in 7% CO_2. Each group was cultured in quadruplicate.

At each end of the incubation time as described above, the T cell cultures were pulsed with 1 μCi of ^{3}H-thymidine per well for 19 to 21 h. Incorporation of ^{3}H-thymidine into the proliferating cells was measured after harvesting the cells on filters (Skatron Microcell Harvester Model 11055) and counting the filters (Beckman Liquid Scintillation Counter Model LS 60001C) in the presence of 4 ml scintillation fluid. Stimulation index (S.I.) was defined as the ratio of counts per min (CPM) of the *in vitro* peptide stimulated cultures over the peptide-free cultures.

Separate parallel T cell cultures under identical conditions were set up for the collection of cytokines produced during T cell proliferation. Culture supernatants were collected from separate plates at 24 h, 48 h and 72 h.

2. Cytokine Analysis

Levels of INF-γ and IL-4 in the T cell cultures were separately estimated at 24 h, 48 h or 72 h by a sandwich-type enzyme-linked immunosorbent assay (ELISA) (118, 119).

The 96-well Nunc-maxisorp ELISA microplates were incubated with a specific amount of antibodies R46.A2 or 11B11 (for INF-γ or IL-4, respectively) at 37°C for 30 min. After washing with TPBS (PBS with 0.05% of Tween 20, pH 7.4), T cell culture supernatants or the reference

standards (rIFN-γ, recombinant IFN-γ : concentration range of 5000-156 pg / ml; rIL-4, recombinant IL-4 : concentration range of 1666-52 pg / ml) were added and incubated at 37°C for 45 min. Followed by washing with TPBS, a specific amount of second antibodies (XMG1.2 biotinylated for IFN-γ assay or BVD6-24G2 biotinylated for IL-4 assay) was added to each corresponding plate and incubated for 45 min at 37°C.

After washing for three times with TPBS, the plates were incubated with peroxidase conjugated streptavidin at 37°C for 30 min, followed by washing in TPBS for four times. TMB peroxidase substrates were then added to each well and the plate was read by a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) at a wavelength of 650 nm. A final reading was taken at 450 nm after adding phosphoric acid into the plates. Cytokine levels in T cell culture supernatants were determined based on the standard curves on each plate.

3. MUC1 Specific Antibody Isotype Analysis

Each of the 96-well Nunc-maxisorp ELISA microplates was coated with a specific amount of BP16 conjugated HSA (human serum albumin) and HSA (negative control) at 4°C over-night. After washing with PBS (pH 7.4), the microplates were incubated for 30 min at 37°C with 200 µl/well BSA (KPL concentrate) in a dilution of 1/10 (v / v) in ddH_2O, followed by washing (3 times) with TPBS (PBS with 0.05% of Tween 20, pH 7.4).

Serum aliquots collected from each mouse of the same group of mice immunized with a certain MUC1 peptide formulation were pooled together for the titration. Test, normal and positive control sera (serial dilutions for IgM: 1/80 to 1/5120 and IgG: 1/2500 to 1/160,000) as well as PBS (plate control) were added at 100 μl/well.

After incubation for 1 h at room temperature, the microplates were washed (5 times) with TPBS. Second antibodies of peroxidase labeled goat anti-mouse IgG and IgM antibodies or horseradish peroxidase labeled goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 antibodies with a specific dilution in PBS (pH 7.4) were added at 100 μl/well.

After incubating at room temperature for 1 h and washing (6 times) with TPBS, ABTS substrates were added to each microplate and the absorbance optical density (O.D.) readings from a microplate reader (Molecular Devices Corporation, Sunnyvale, CA) were taken at 405 nm at the 15 min end points.

I. Statistical Analysis

Values are expressed as means ± standard deviations of three experiments, otherwise as mentioned. Statistical differences were established by analysis of variance (ANOVA) and student's t-test. Levels of $P < 0.01$ were considered to be significant.

III. RESULTS

A. Peptide Quantification

The quantity of peptide encapsulated in liposomes was of interest in the present study. Therefore, it was important to develop a direct method for quantification of peptides in liposomal pellets.

1. BCA Assay

Preliminary results of BP30 peptide quantified by the BCA assay showed low sensitivity due to a very high background (Figure 6).

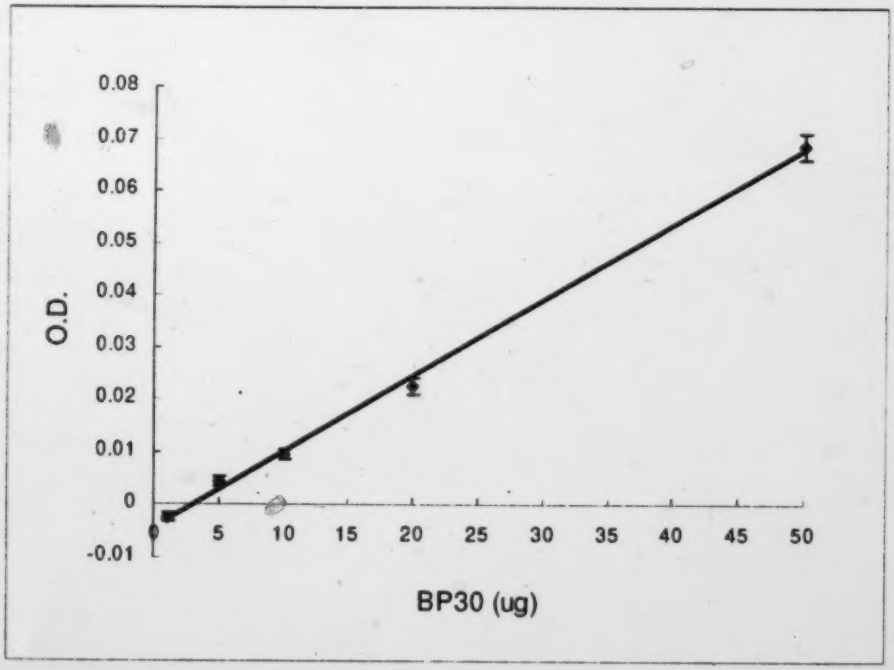

Figure 6. Calibration curve of BP30 peptide with background subtracted as determined by BCA assay. Optical density (O.D.) was read at a wavelength of 540 nm. Values are expressed as means ± range, n=3.

Lipid interference in the peptide BCA assay was studied by adding empty DMPC liposomes into a known amount of the MUC1 peptide BP30 at a ratio of 20:1 (w / w). Results showed that the liposomes interfered with peptide absorbance significantly (Figure 7).

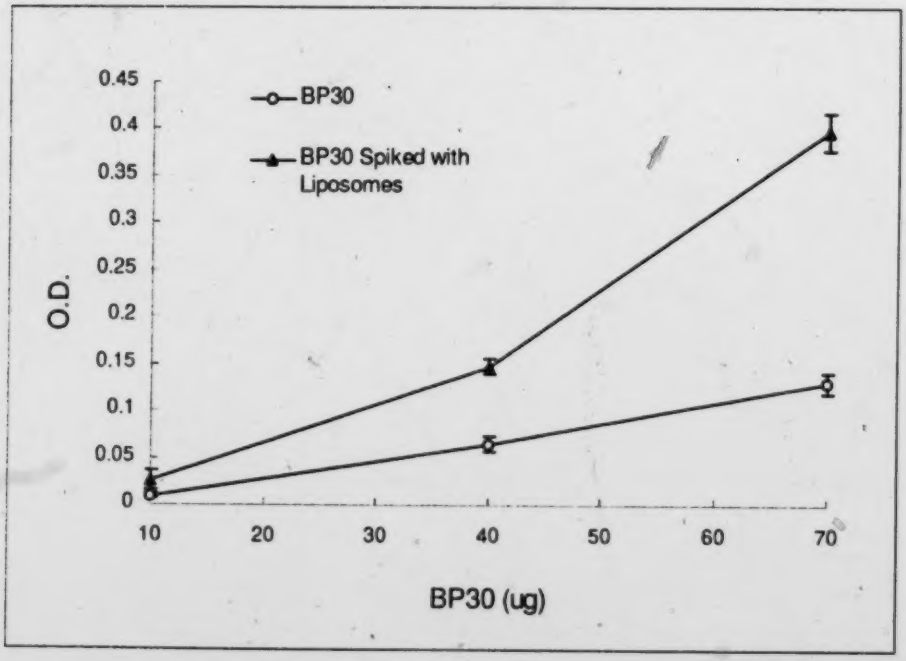

Figure 7. Comparison of absorbance of BP30 peptide alone and spiked with empty liposomes, by the BCA assay. Optical density (O.D.) was read at a wavelength of 540 nm.

2. HPLC Analysis

The HPLC method was found to be reliable and reproducible for MUC1 peptides in buffer or in liposomes. A sample HPLC elution profile of the peptide in PBS is shown in Figures A-1 to A-3 of the Appendix. A

sample calibration curve of BP24 is shown in Figure 8 (see Figures B-1 to B-3 of the Appendix for the rest of the standard curves for MUC1 peptides). Reliability of the method was demonstrated in that less than a 5% difference was found after 6 months.

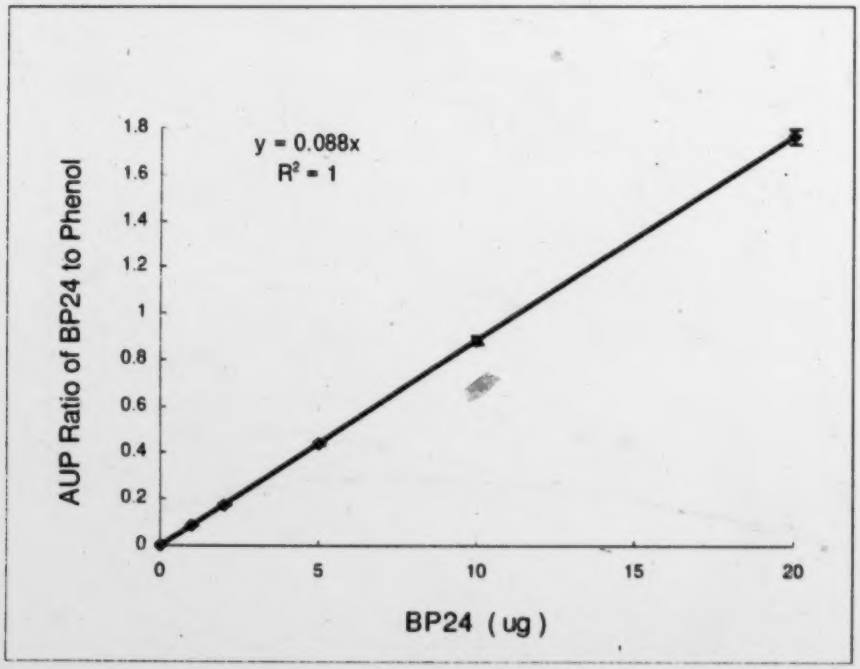

Figure 8. Calibration curve of BP24 by the HPLC method. AUP stands for area under the peak . Phenol was used as the internal reference. Values are expressed as means ± range, n=3.

When Triton X-100 was used to dissolve the liposomal pellets, an additional broad absorption peak from Triton X-100 (2.5%, v / v) appeared at the end of the analysis (Figure 9). Residues of Triton X-100 had to be removed prior to the next injection, otherwise an interfering

slanted baseline would have resulted. Therefore, the time of analysis was prolonged by 40% (35 min) compared to analysis of peptide in buffered solution (25 min).

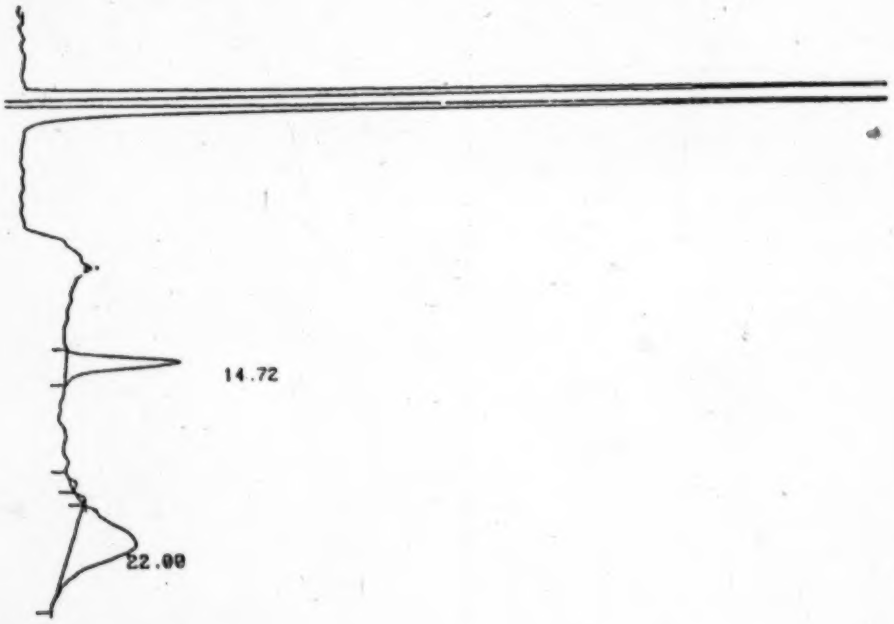

Figure 9. Effect of Triton X-100 (retention time: RT, 22 min) on the elution profile of phenol (RT, 14.72 min) on a C-18 reversed phase radial-pak cartridge (8 mm x 100 mm). Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (96% to 54% A in 14 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

To improve the HPLC assay, efforts were made to find a better solvent for the liposomal pellets. Among several solvents, absolute ethanol was found to be the best for dissolving the pellets and recovering

the peptides, as well as having a reliable HPLC profile. Table II shows the recovery of a known amount of BP24 from spiked empty liposomes and various amounts of absolute ethanol. The recovery decreased when the concentration of ethanol was higher than 60% (v / v). Recovery was found to be 100 ± 3.7%.

Table II. Recovery of BP24 from peptide spiked with empty DPPC liposomes and various amount of absolute ethanol

	Ethanol (v / v)	Recovery (%)
BP24	40%	100 ± 2.4
	60%	89 ± 2.1
	70%	76 ± 1.9
BP24 Spiked with Pellets	20%	100 ± 2.0
	40%	100 ± 1.8
	60%	90 ± 2.2

Example HPLC elution profiles corresponding to Table II are shown in Figure A-1 and A-2 of the Appendix. Specifically, after empty liposomal pellets were dissolved in absolute ethanol, known amounts of BP24 (1 μg / 50 μl) and phenol were added, mixed and analyzed by HPLC (Appendix Figure A-1 and A-2).

Figure 10 shows a sample HPLC profile of liposome encapsulated peptide. Each analysis of liposomal pellets with ethanol lasted 22 min which was the same as the peptide in buffered solution.

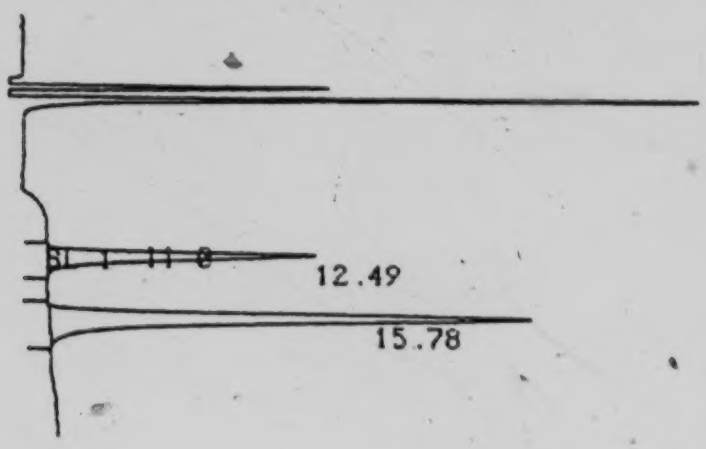

Figure 10. Elution profile of BP24 in liposomes dissolved in absolute ethanol (BP24 RT, 12.49 min) with internal reference phenol (RT, 15.78 min) on a C-18 reversed phase radial-pak cartridge (8 mm x 100 mm). Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (96% to 54% A in 14 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

Mass balance was verified from peptide quantities in liposomal pellet and supernatant of liposome samples taken at various time intervals during incubation at 37^0C or storage at 4^0C. Table III shows an example of the mass balance data.

Table III. Mass balance recovery of BP24 in DPPC liposomes during 31 day storage at 4°C. BP24 quantities were based on HPLC analyses of the peptide in supernatant and in liposomal pellet.

Day	0	3	9	16	24	31
BP24 in supernatant (μg)	58±2.3	58±2.1	58±1.8	59±2.4	58±2.1	59±2.3
BP24 in pellet (μg)	31±1.4	30±1.1	29±1.3	30±1.1	29±1.2	29±1.5
Recovery	89%	88%	87%	89%	87%	88%

Note: aliquot liposome sample of 200 μl contained 100 μg BP24.

B. Liposome Characteristics

1. Encapsulation Efficiency as a Function of Charge, pH, Phospholipid Composition and Ionic Strength

The encapsulation efficiency of MUC1 peptides BP24 or SP1-020 in liposomes as a function of pH or liposome charge is described in Table IV. The encapsulation efficiency of BP24 or SP1-020 was 5 to 7-fold higher ($P<0.01$) in charged than in neutral liposomes of DPPC:CHOL (3:1 mole ratio). The encapsulation efficiency was essentially independent of the buffer pH from 4 to 7.4 in each case ($P>0.01$).

Table IV. Encapsulation efficiency (%) of BP24 or SP1-020 in liposomes of 0.2 M ionic strength as a function of liposome charge and pH of the medium.

pH of Medium	Neutral DPPC:CHOL (3:1)	Negative DPPC:CHOL:DMPG (3:1:0.25)	Positive DPPC:CHOL:SA (3:1:0.25)
4.0	5.2 ± 0.6	33 ± 2.4	27 ± 1.4
5.5	4.3 ± 0.8	35 ± 1.5	26 ± 2.3
7.4	5.0 ± 0.7	35 ± 1.8	29 ± 2.0
7.4[a]	6.1 ± 0.5	33 ± 1.4	31 ± 2.2

[a] SP1-020

In negatively-charged liposomes at 0.1 M ionic strength, a maximum encapsulation efficiency was attained with as little as 6 mole percent (0.25 mole ratio) DMPG (Figure 11). DMPC:CHOL liposomes had only half of the encapsulation efficiency of the longer chain phosphatidylcholine liposomes.

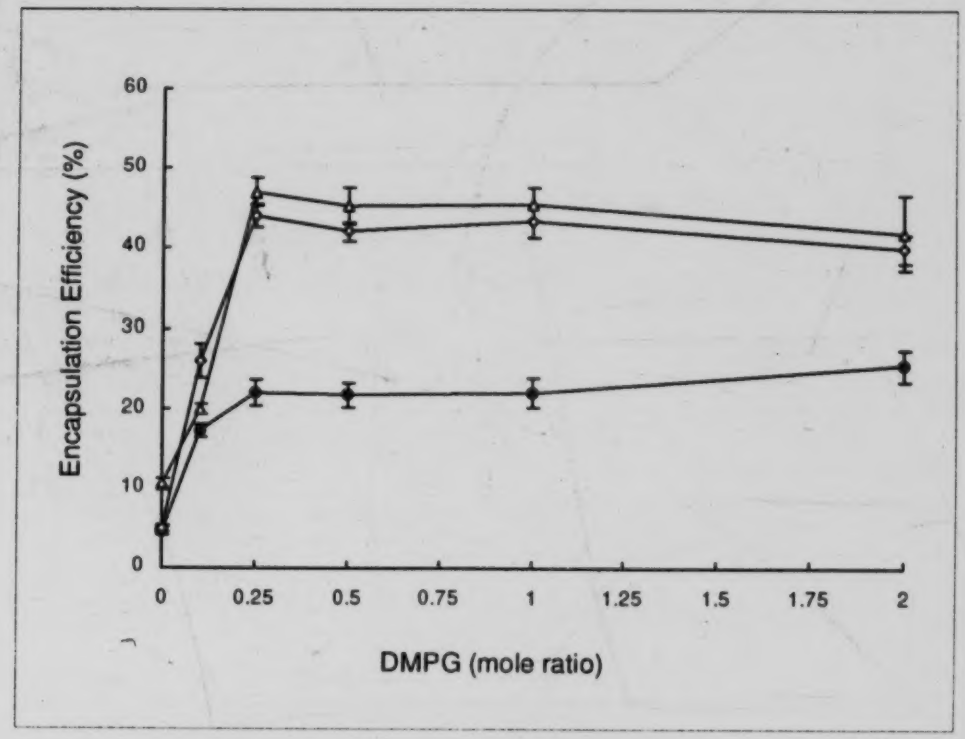

Figure 11. Encapsulation efficiency of BP24 in DSPC:CHOL (–Δ–), DPPC:CHOL (–◊–) and DMPC:CHOL (–•–) liposomes (3:1 mole ratio) as a function of DMPG at pH 7.4 and 0.1 M ionic strength.

The encapsulation efficiency of BP24 or SP1-020 was also affected by the ionic strength of the dispersion medium (Figure 12 and 13). Encapsulation efficiency was significantly improved ($P<0.01$) by reducing the ionic strength from 0.4 M to 0.04 M. The encapsulation efficiency was independent of the buffer type used, i.e. PBS or Tris ($P>0.01$) (Figure 12).

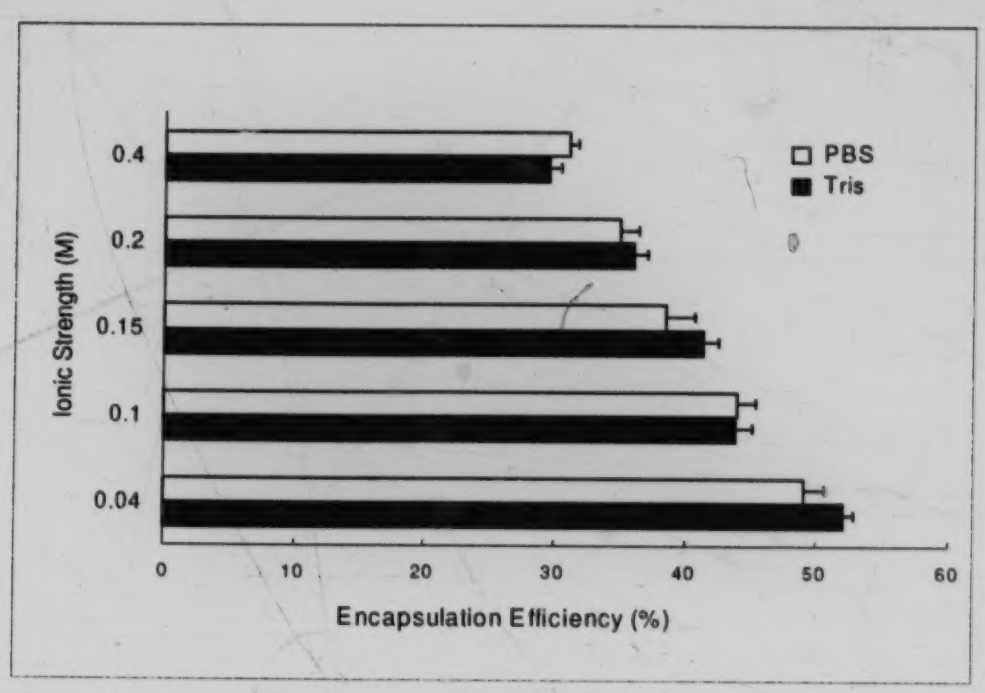

Figure 12. Encapsulation efficiency of BP24 in DPPC:CHOL:DMPG (3:1:0.25 mole ratio) liposomes as a function of ionic strength at pH 7.4 in PBS or Tris buffer system.

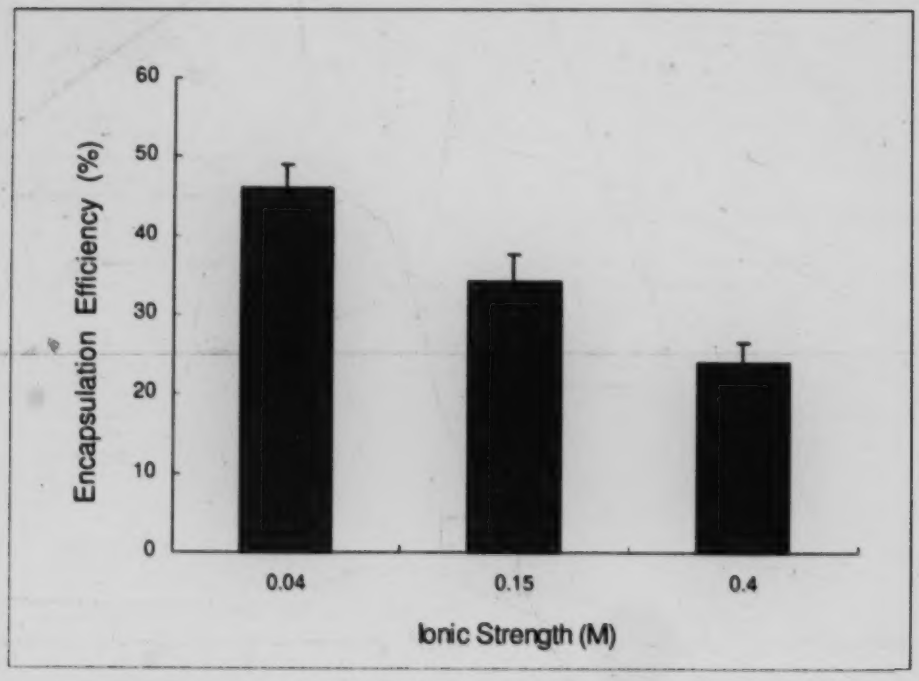

Figure 13. Encapsulation efficiency (%) of SP1-020 in DSPC:CHOL:DMPG (3:1:0.25 mole ratio) liposomes as a function of ionic strength in pH 7.4 PBS.

Incorporation of MPLA in liposomes did not significantly alter the encapsulation efficiency of BP24 ($P>0.01$) at a range of MPLA from 0.05% to 2% (w/w, MPLA / lipids) (Figure 14).

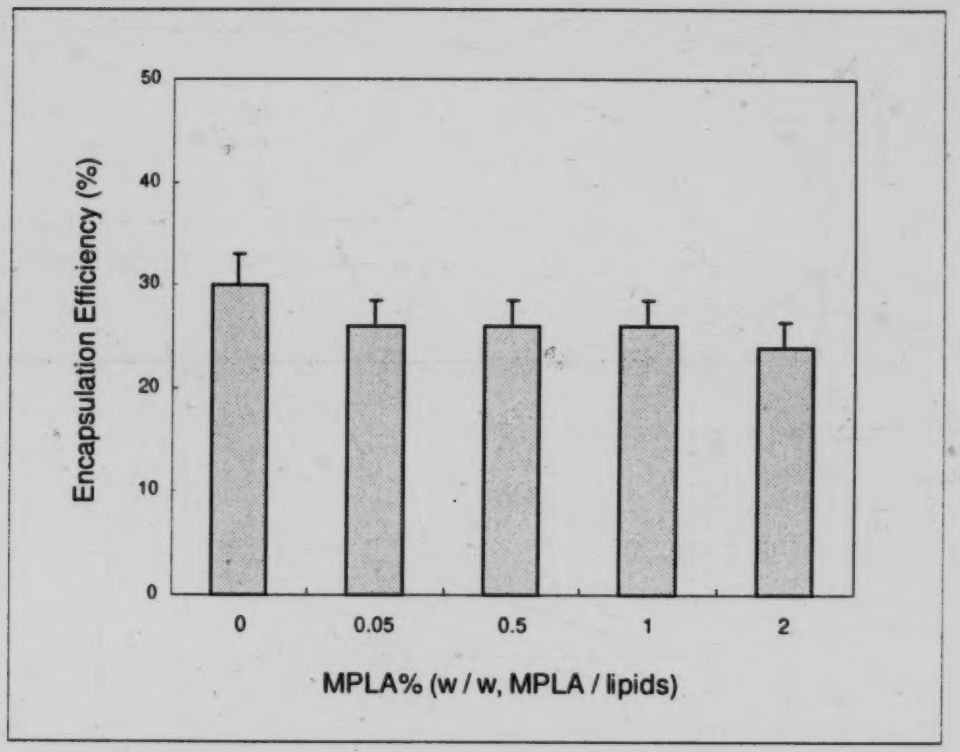

Figure 14. The encapsulation efficiency of BP24 as a function of various amounts of MPLA in liposomes of DPPC:CHOL:DMPG, 3:1:0.25 mole ratio.

2. Peptide Retention

a. In PBS

Studies of the effect of phospholipid composition on BP24 peptide retention showed no significant leakage found with DSPC or DMPC liposomes whereas the peptide was retained only 85% in DPPC liposomes at 37°C (Figure 15). However, there was no difference in BP24 peptide retention among those liposomes after 31 day storage at

4°C (Figure 16). The stored liposomes were found to be stable for more than a year, since more than 85% of the peptide remained within the liposomes (Figure 17). Both hydrophilic peptide BP25 and its amphiphilic analogue BLP25 were retained to more than 90% at 37°C for 24 h (Figure 18).

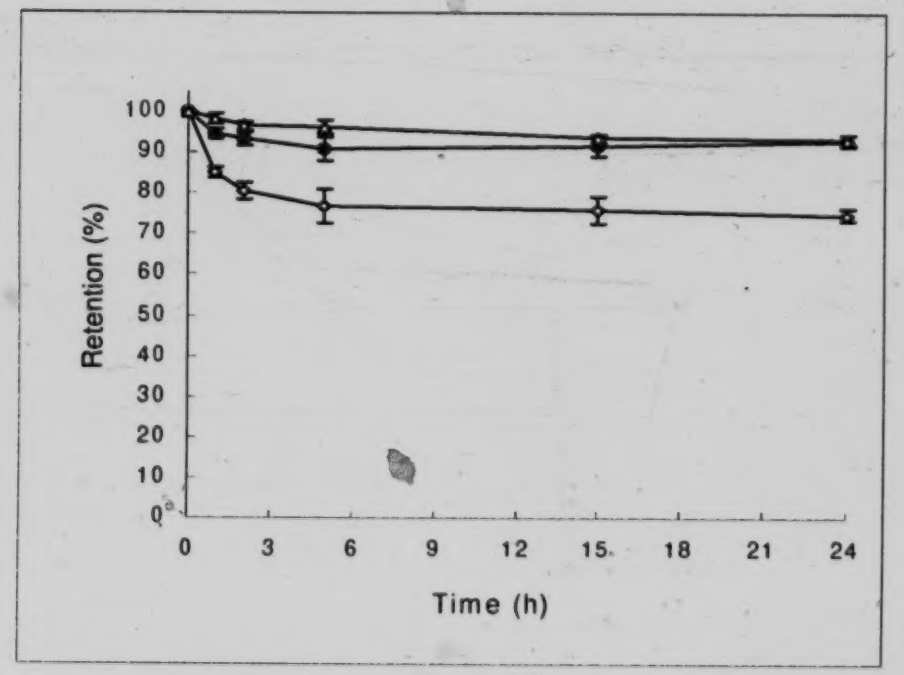

Figure 15. Retention (%) of BP24 in liposomes of various phospholipid compositions: DSPC (–Δ–), DPPC (–◊–) or DMPC (–•–) as a function of incubation time at 37°C.

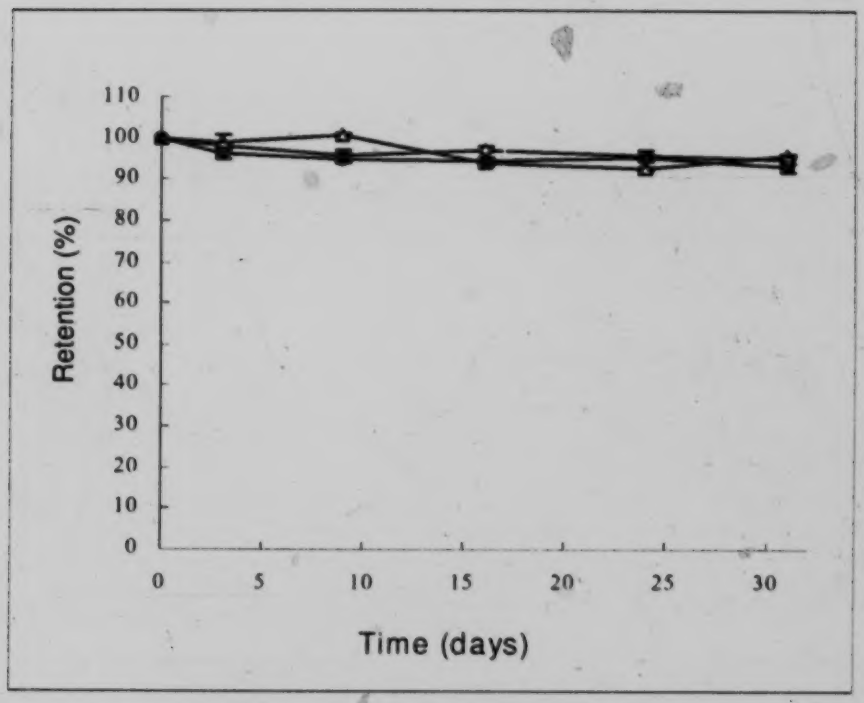

Figure 16. Retention (%) of BP24 in liposomes of various phospholipid compositions: DSPC (-Δ-), DPPC (-◊-) or DMPC (-•-) as a function of storage time at 4°C.

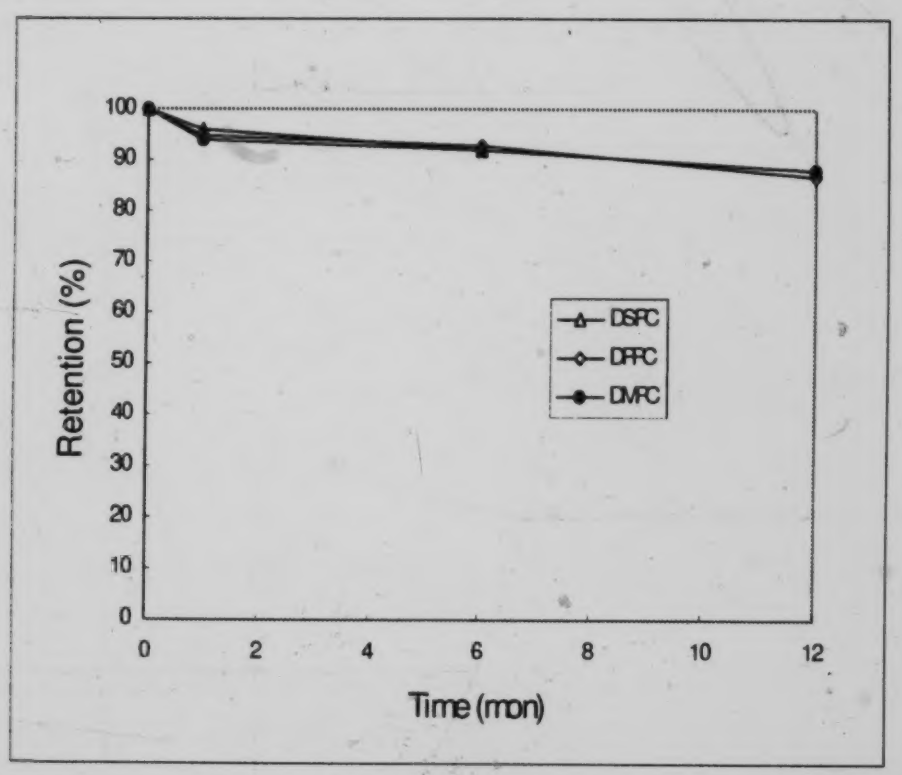

Figure 17. Retention (%) of BP24 in liposomes of various phospholipid compositions as a function of storage time at 4°C.

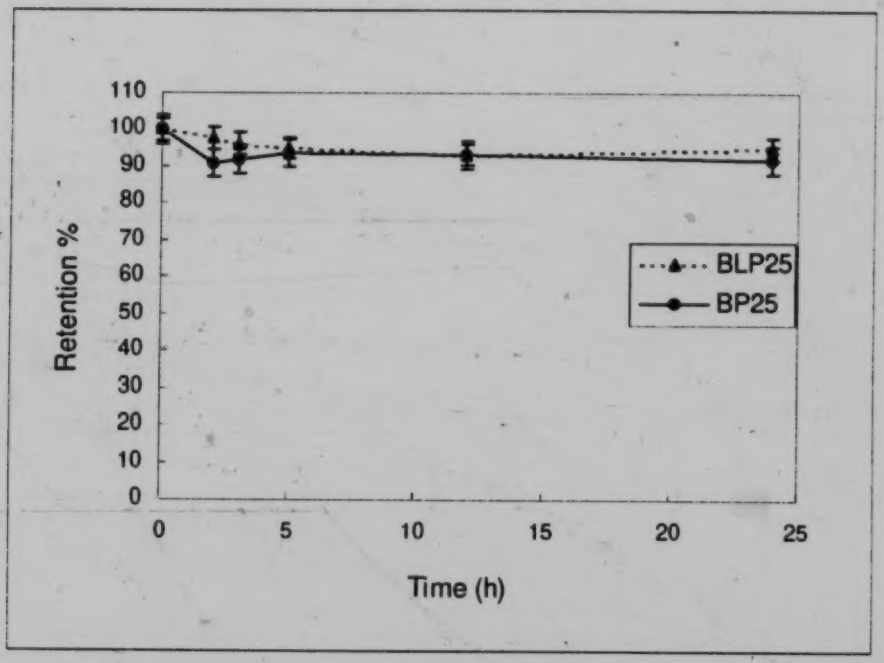

Figure 18. Retention (%) of BP25 and its acylated analogue BLP25 in liposomes composed of DSPC:CHOL:DMPG (3:1:0.25), when incubated at 37°C for 24 h.

b. In Serum

Figure 19 shows the HPLC analysis of liposomes containing mouse MUC1 SP1-020 incubated in 100% mouse serum at 37°C for 18 h. The sharp HPLC profile was obtained after two washes of the serum-incubated liposomes. More than 80% of the SP1-020 was retained in DSPC, DPPC, DMPC:CHOL:DMPG (3:1:0.25, mole ratio) liposomes (Figure 20).

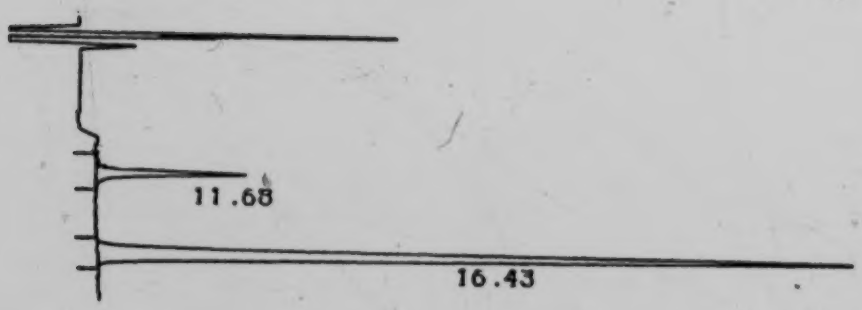

Figure 19. Elution profile of SP1-020 liposomes incubated in 100% mouse serum at 37°C after 18 h (RT, 11.68 min). The HPLC sample was prepared with phenol as internal reference (RT, 16.43 min) after dissolving the liposomal pellet with absolute ethanol. Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (100% to 60% A in 15 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

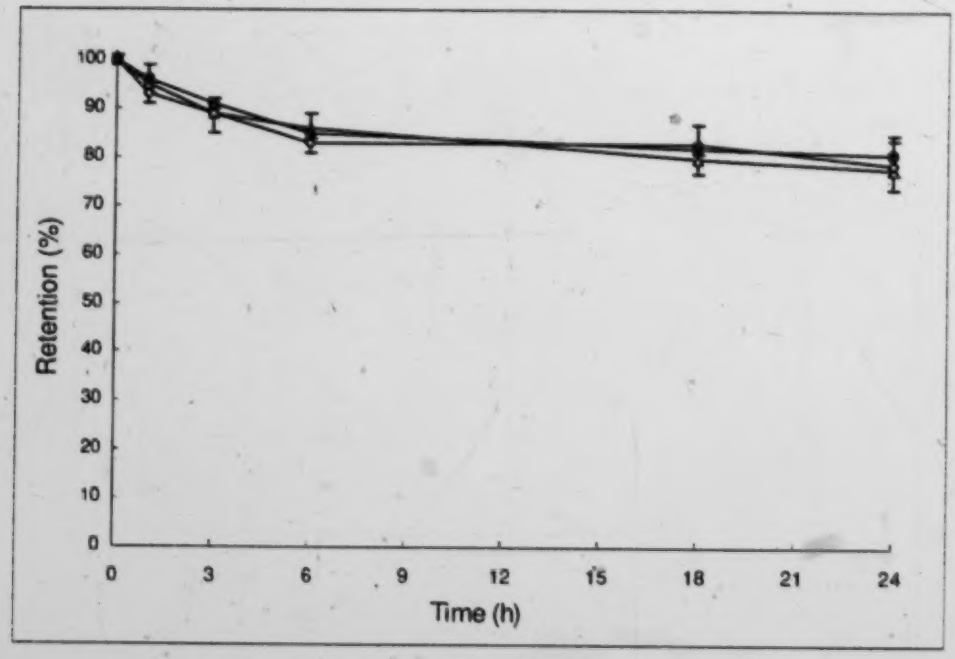

Figure 20. Retention (%) of mouse MUC1 peptide SP1-020 in liposomes of various phospholipid compositions: DSPC (–Δ–), DPPC (–◊–) or DMPC (–•–) as a function of incubation time at 37°C in 100% mouse serum.

Figure 21 shows an HPLC chromatogram from the analysis of liposomes containing an amphiphilic human MUC1 BLP25 incubated in 100% human serum at 37°C for 18 h. Similar results were obtained for 100% human serum incubated liposomes containing human MUC1 BP25 and BLP25 peptides. Greater than 75% of peptide were retained in liposomes after 24 h incubation at 37°C (Figure 22).

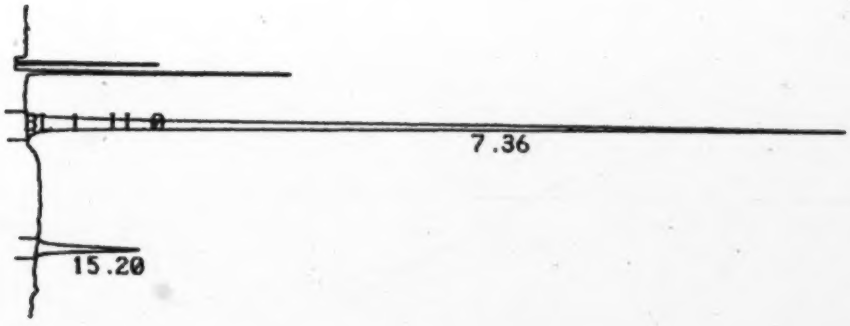

Figure 21. Elution profile of BLP25 liposomes incubated in 100% human serum at 37°C after 18 h (RT, 15.20 min). The HPLC sample was prepared with phenol as internal reference (RT, 7.36 min) after dissolving the liposomal pellet with absolute ethanol. Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (81% to 39% A in 15 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

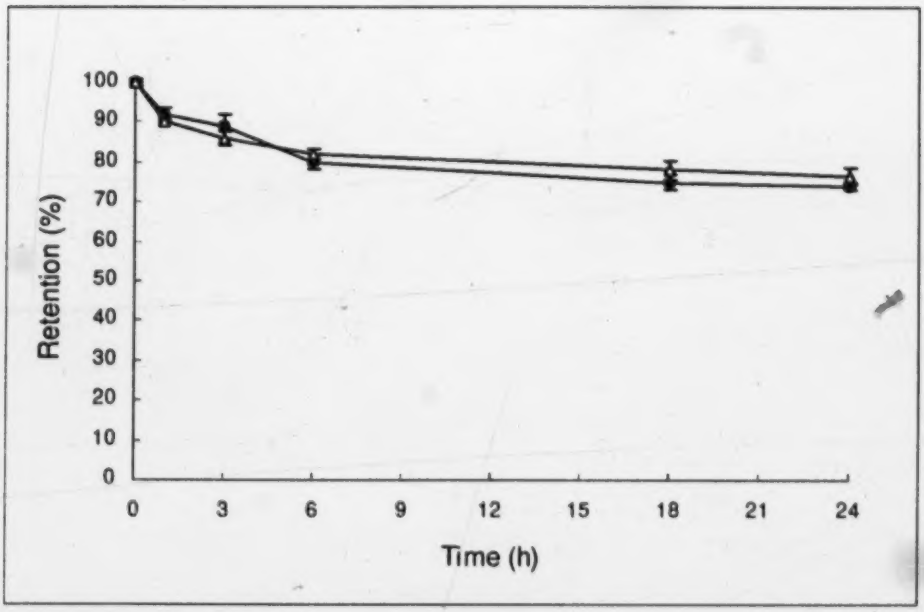

Figure 22. Retention (%) of human MUC1 peptides BP25 (–Δ–) and BLP25 (–•–) in DSPC:CHOL:DMPG (3:1:0.25, mole ratio) liposomes as a function of incubation time at 37°C in 100% human serum.

Mass spectrometric analyses showed that the molecular weight of BLP25 was not significantly changed (2628.03 ± 1.81 prior to incubation, Figure 23, versus 2625.92 ± 0.20 at hour 24, Figure 24) after incubation of BLP25 liposomes in 100% human serum at 37°C. The rest of the mass spectrometric analyses at hour 3, 6 and 18 are included in the Appendix (Figure D-1 to D-3).

Results liposome sizing after incubation in 100% serum at 37°C showed that the size was not significantly changed for at least 24 h, indicating intact liposomes. For example, at 0 h, the average size was 1079 nm ± 110 nm and at 24 h, 1093 nm ± 143 nm.

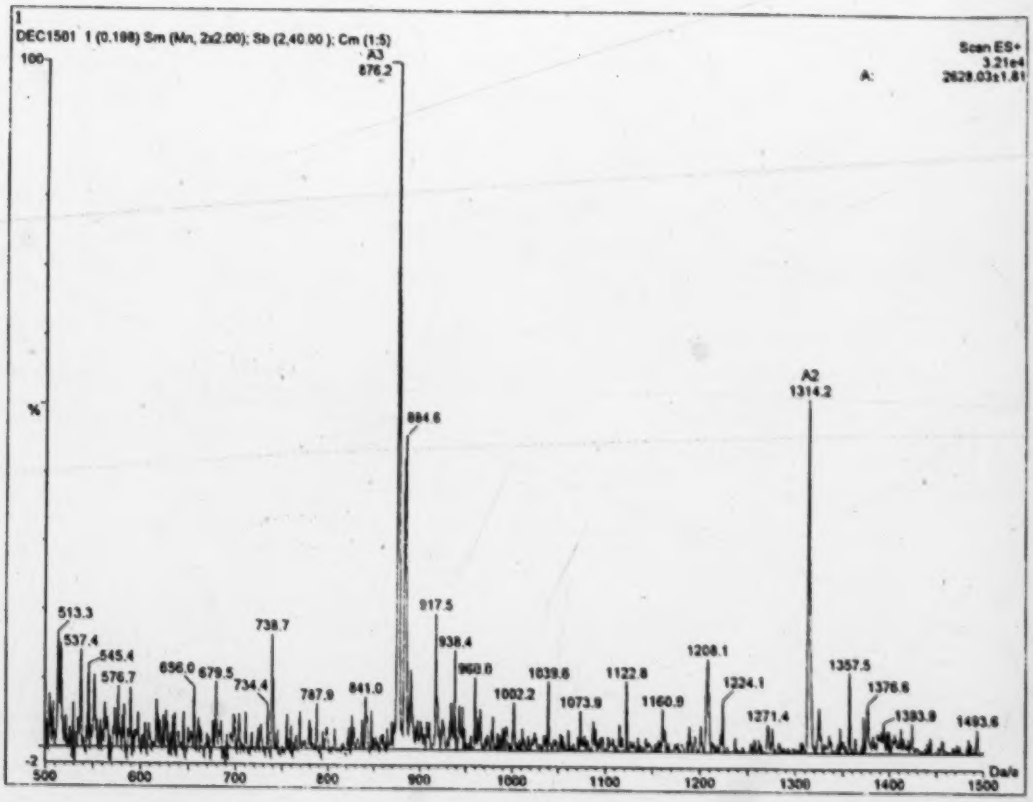

Figure 23. Mass spectrum (at 79°C and 46 voltage) of BLP25 collected from HPLC pellet analyses of BLP25 liposomes prior to the incubation with 100% human serum. The molecular weight of BLP25 (A: 2628.03 ± 1.81) was generated based on the molecules carrying 2 charges (A2: 1314.2) and 3 charges (A3: 876.2) as shown.

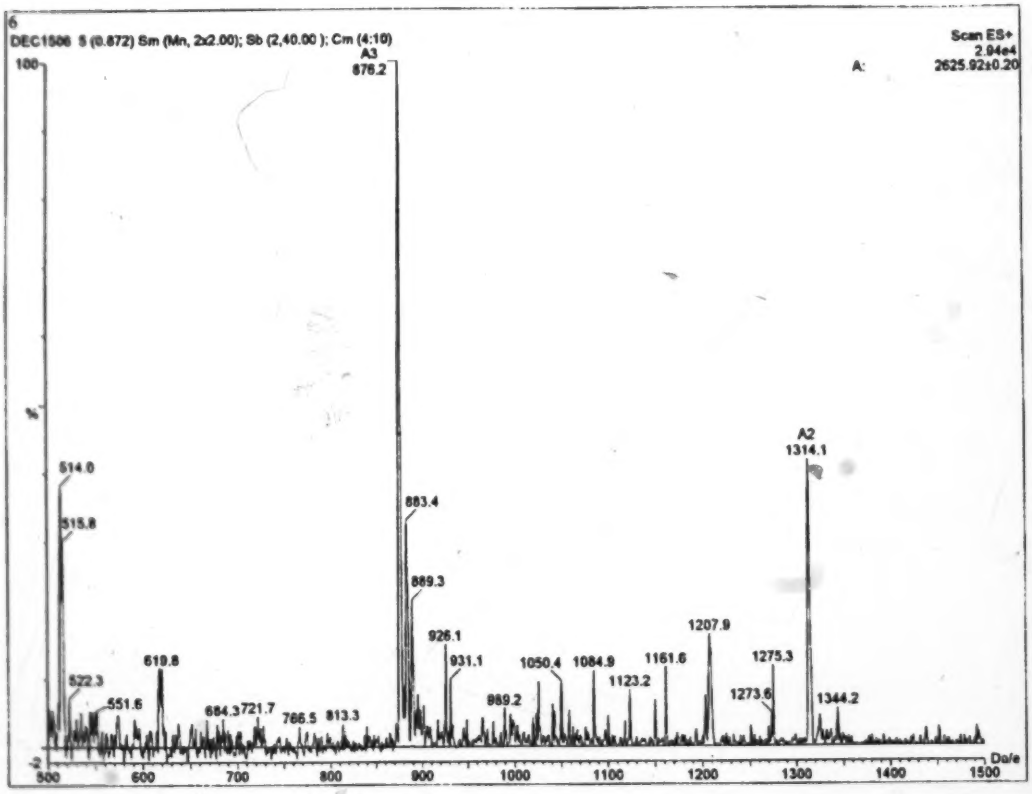

Figure 24. Mass spectrum (at 79°C and 46 voltage) of BLP25 collected from HPLC pellet analyses of BLP25 liposomes incubated for 24 h with 100% human serum at 37°C. The molecular weight of BLP25 (A: 2625.92 ± 0.20) was generated based on the molecules carrying 2 charges (A2: 1314.1) and 3 charges (A3: 876.2) as shown.

3. Liposome Surface Exposure of MUC1 Epitopes

Liposomes incorporated with lipid-conjugated (BLP25, BLP24) and non-conjugated (BP25, BP24) MUC1 peptides were characterized for surface exposure of MUC1 epitopes by flow cytometry. FACS (fluorescence activated cell sorter) analyses of the liposome samples included 'empty liposomes', BP25 mixed with 'empty liposomes' and BP25- or BLP25-entrapped liposomes ('liposomal BP25' and 'liposomal BLP25') with a MUC1 specific mAb B248.7R2 (specific for the amino acid sequence APPAHGVTSA of the human MUC1 mucin) showed that the MUC1 specific epitopes were only present on the surfaces of 'liposomal BLP25' (N-terminal acylated MUC1 peptide) preparations (Figure 25). An irrelevant mAb B80.3 (specific for PSA) did not bind with 'liposomal BLP25', establishing the antigen specificity of the FACS analysis (Figure 25). No MUC1-specific epitopes could be detected on the surfaces in the rest of the liposome formulations (Figure 26). However, when the MUC1 peptide is C-terminal acylated, there also was no detectable MUC1 epitope at liposomal surfaces (Figure 27).

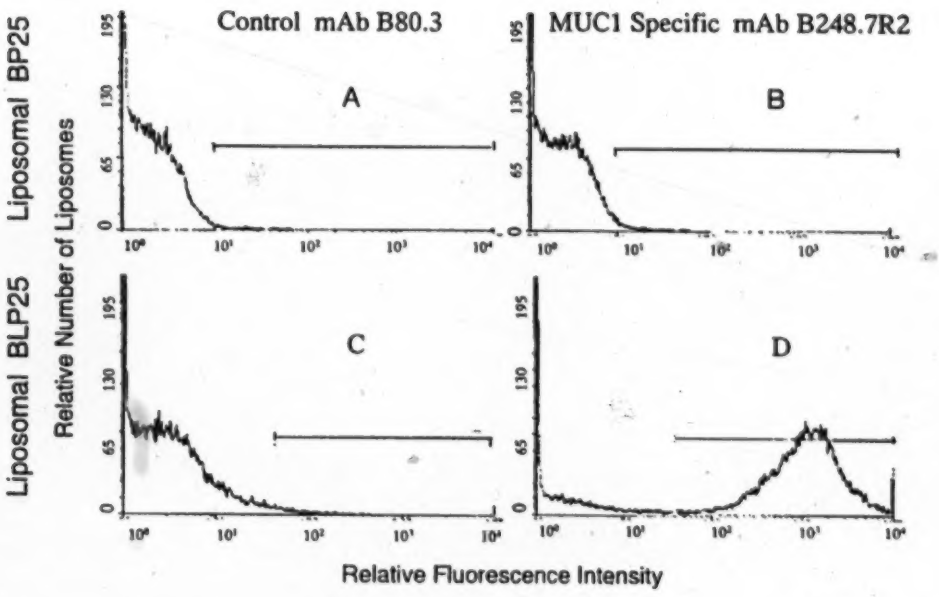

Figure 25. FACS analyses of liposomal BP25 (A, B) and BLP25 (C, D) for presence of MUC1 epitope on the liposome surfaces. Liposomes were incubated with negative control mAb B80.3 specific for PSA (A, C) or MUC1-specific mAb B248.7R2 specific for the amino acid sequence APPAHGVTSA (B, D), subsequently incubated with an appropriate FITC-labeled secondary antibody and analyzed by flow cytometry.

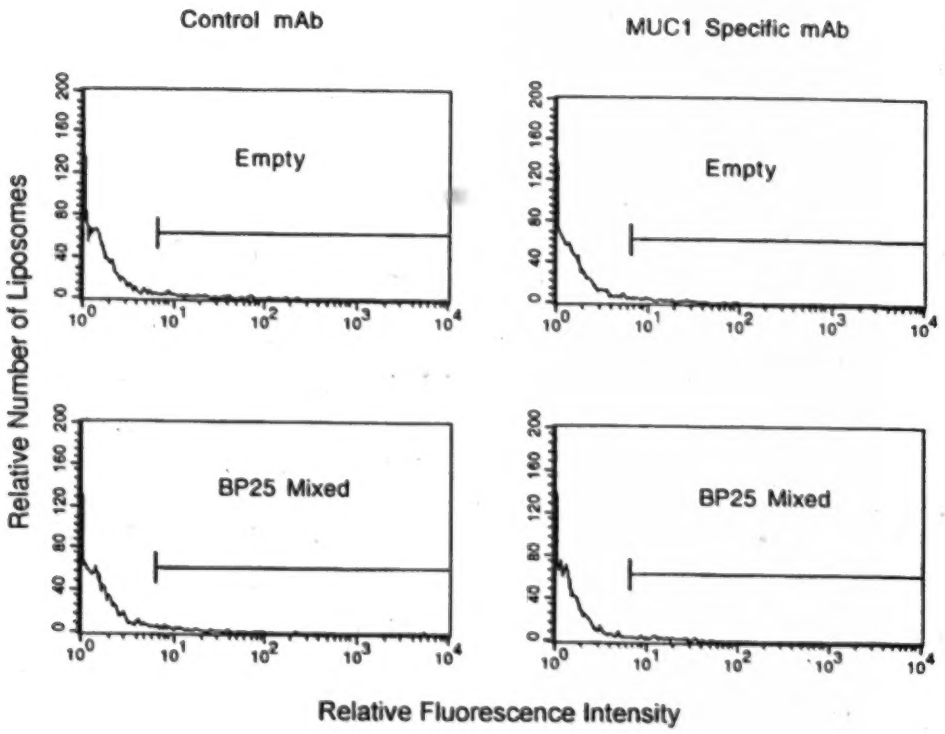

Figure 26. FACS analyses of peptide free 'empty' liposomes and BP25 mixed with 'empty' liposomes for presence of MUC1 epitope on the liposome surfaces. Liposomes were incubated with negative control mAb B80.3 specific for PSA or MUC1 specific mAb B248.7R2 specific for the amino acid sequence APPAHGVTSA, subsequently incubated with an appropriate FITC-labeled secondary antibody and analyzed by flow cytometry.

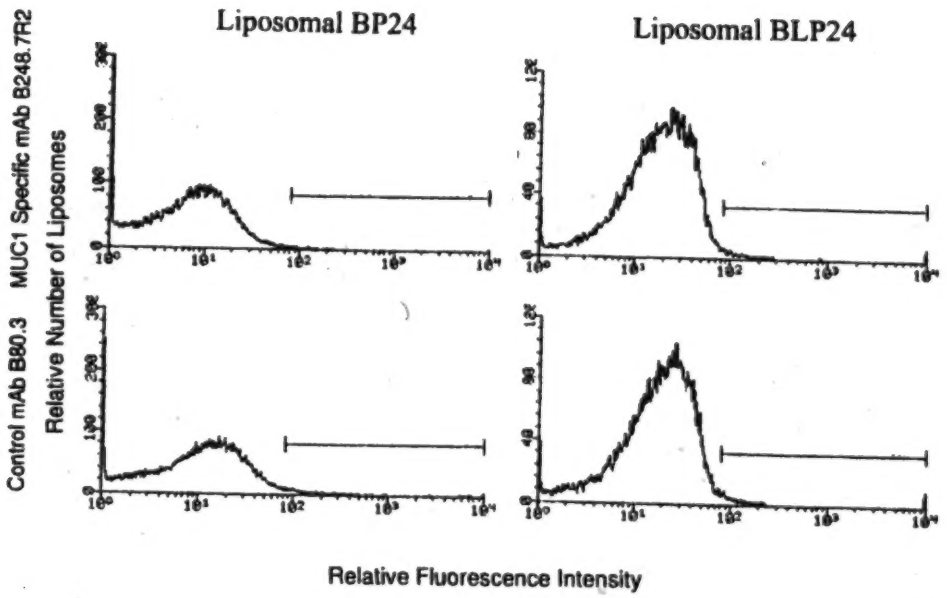

Figure 27. FACS analyses of liposomal BP24 and BLP24 for presence of MUC1 epitope on the liposome surfaces. Liposomes were incubated with negative control mAb B80.3 specific for PSA or MUC1-specific mAb B248.7R2 specific for the amino acid sequence APPAHGVTSA, subsequently incubated with an appropriate FITC-labeled secondary antibody and analyzed by flow cytometry.

C. Effects of Location of MUC1 Peptides in Liposomes on Immune Responses

1. MUC1 Specific T Cell Responses

MUC1-specific T cell responses induced by the formulations differing in the nature of association of BP25 and BLP25 peptides with liposomes were studied by T cell proliferation assays. Results showed that a physical association of the peptide with liposomes was required for induction of strong and specific T cell response.

Human MUC1 specific T cell responses (CPM or S.I.) were significantly stronger (P=0.0005) in groups of mice immunized with peptide-associated liposomes (encapsulated: BP25 liposomes, or surface-exposed: BLP25 liposomes) than the rest of the formulations, either after a single immunization (Figure 28) or after two immunizations (Figure 29). There were no significant differences (P=0.18) in T cell responses (S.I.) between the encapsulated and surface-exposed liposome preparations, or between the two different immunization procedures (Figure 28 and 29). Antigen specificity was established by the observation that T cells responded to both recall human MUC1 peptides BP24 and BP25, but not to the irrelevant mouse MUC1 peptide SP1-020.

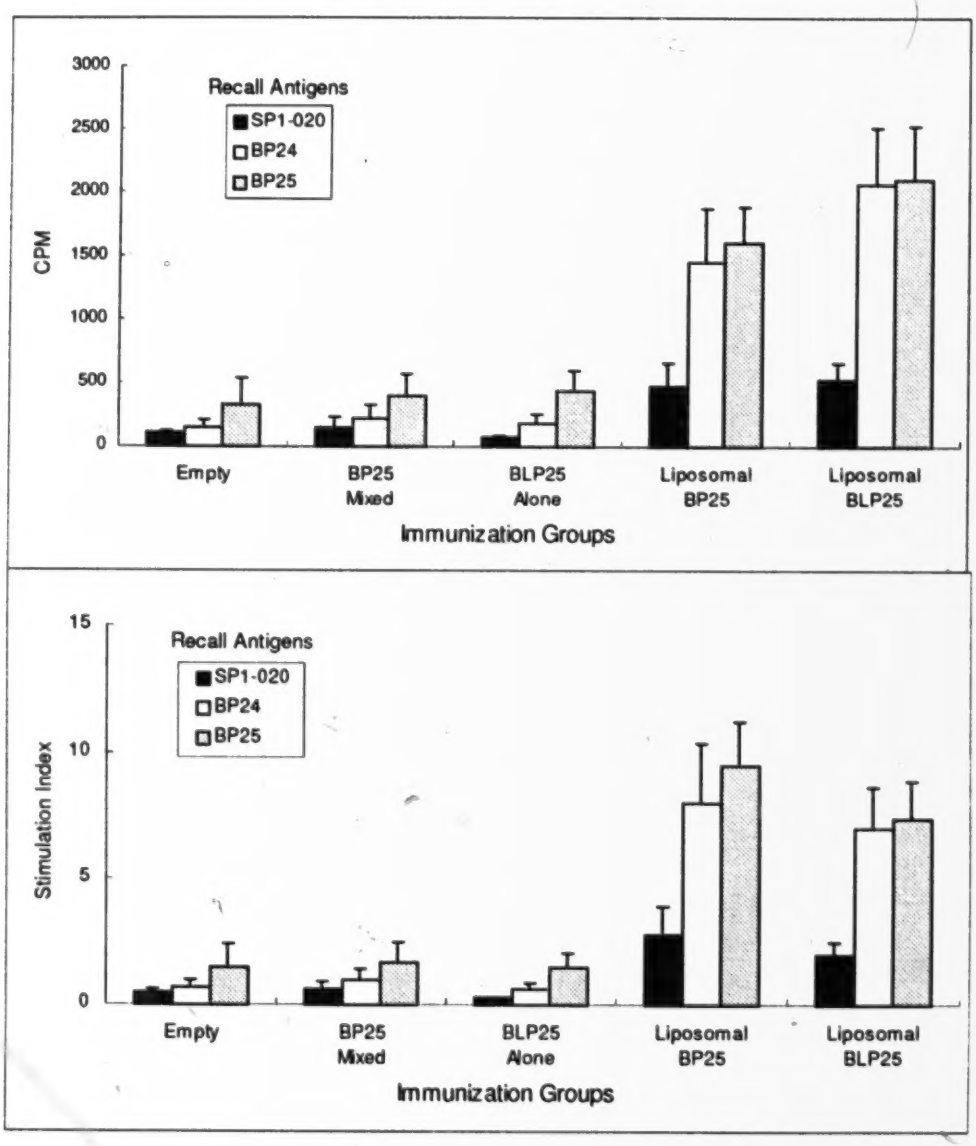

Figure 28. T cell responses induced by five immunization formulations at a dose of 2 nmole peptide per mouse after one immunization in C57BL/6 mice. T cells ($2.5x10^5$/well) purified from the popliteal lymph nodes on day 10 were cultured along with APCs alone or in the presence of human MUC1 peptides or an irrelevant mouse MUC1 peptide, as shown.

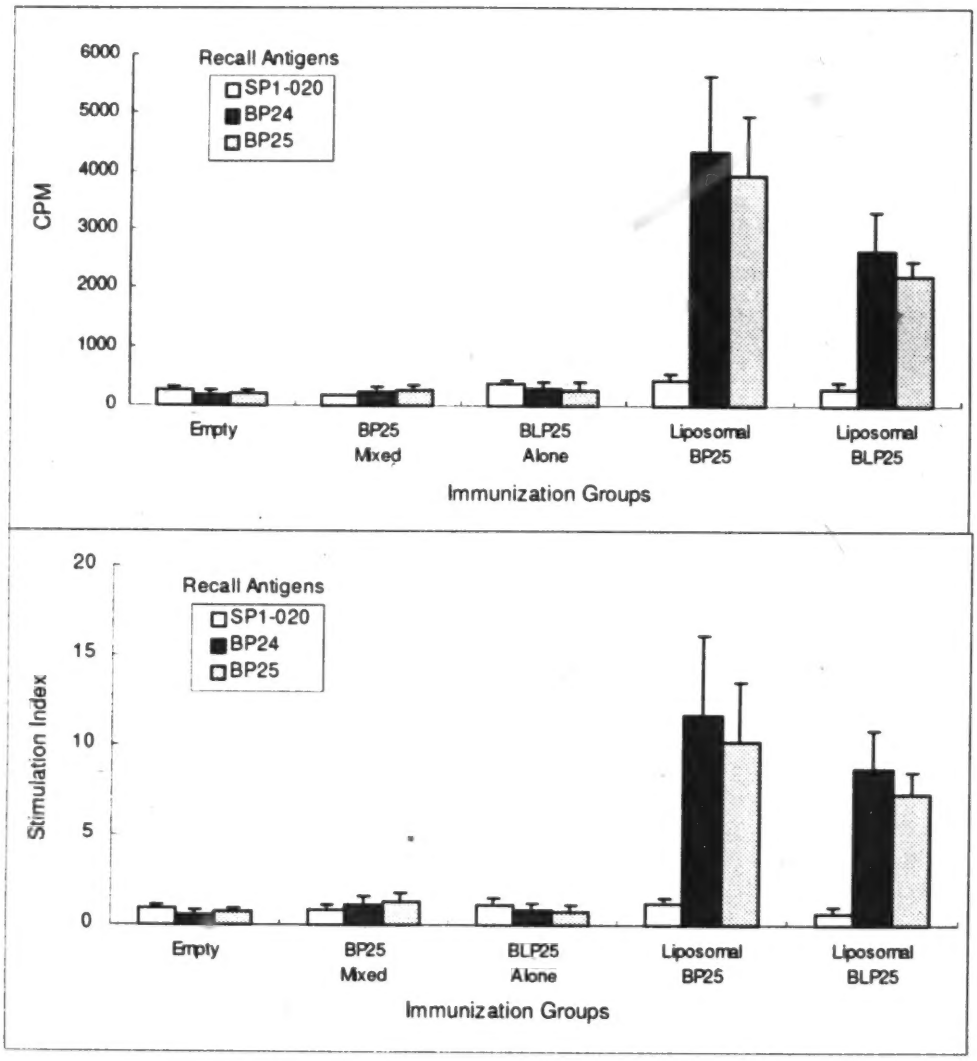

Figure 29. T cell responses induced by five immunization formulations at a dose of 2 nmole peptide per mouse after two immunizations (s.c. and i.p.) in C57BL/6 mice. T cells (2.5×10^5/well) purified from the inguinal lymph nodes on day 26 were cultured along with APCs alone or in the presence of human MUC1 peptides or an irrelevant mouse MUC1 peptide, as shown.

2. MUC1 Specific Humoral Immune Responses

a. In C57BL/6 Mice

MUC1-specific humoral immune responses were evaluated by ELISA using BP16-HSA as the solid phase. BP16 contains the immunodominant B cell epitopes of MUC1 tandem repeat sequences in mice (71). The positive control serum for IgG and IgM was from mice immunized with BP16-KLH emulsified in RIBI DETOX adjuvant.

There was no detectable level of IgG or IgM after one immunization in any of the groups of mice immunized with the five BP25 or BLP25 preparations (Figure 30). However, high titers of both IgG and IgM were detected in the sera pooled from the group of mice immunized twice with the 'liposomal BLP25' preparation, only, and not from any other groups of mice immunized with the remaining four formulations (Figure 31).

These sera were further analyzed for IgG antibody isotype presence and distribution. Results of MUC1 specific IgG isotype analyses for IgG1, IgG2a, IgG2b and IgG3 showed a domination of IgG2b over IgG1 (94% versus 6%). There was no detectable level of IgG2a or IgG3 in the sera (Figure 32).

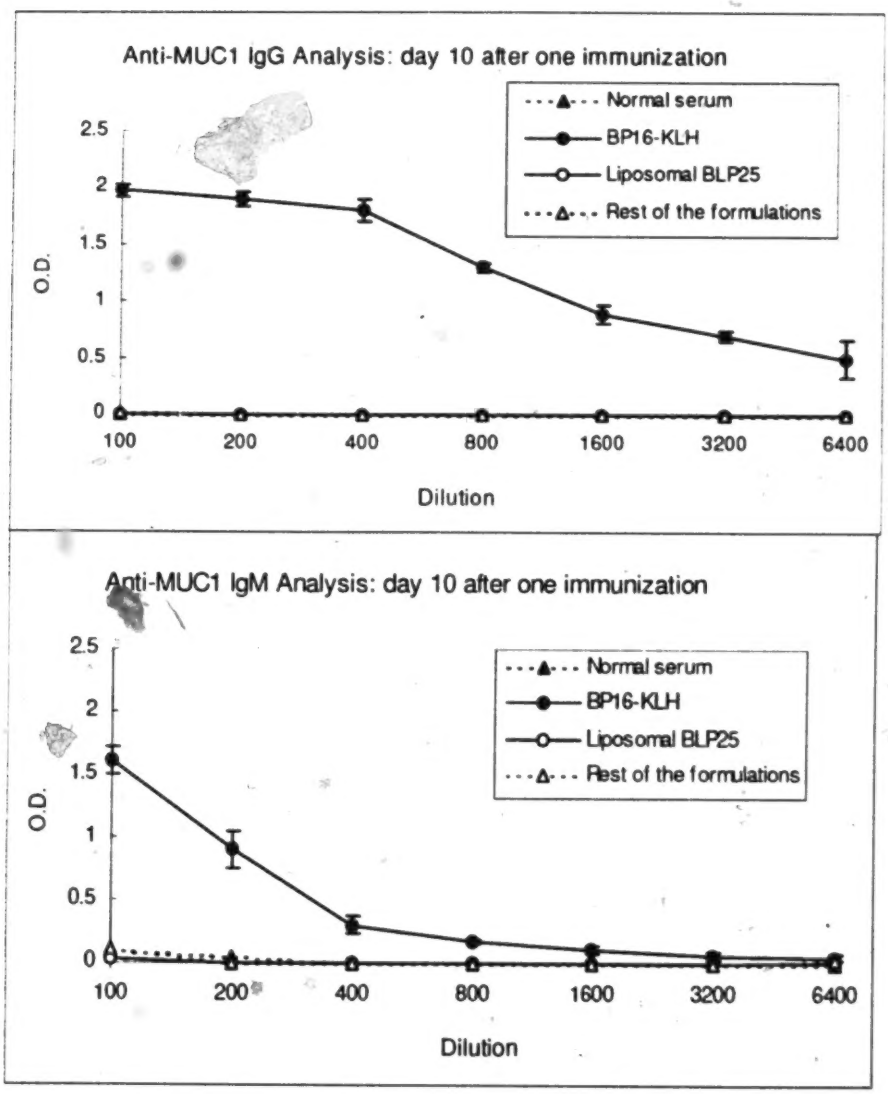

Figure 30. Anti-MUC1 IgG and IgM analyses from sera pooled from each group of the C57BL/6 mice immunized (s.c.) with the five formulations: 'Empty', 'BP25 mixed', 'BLP25 alone', 'Liposomal BP25' and 'Liposomal BLP25'. Polyclonal antiserum from BP16-KLH immunized mice served as the positive control. Optical density (O.D.) was read at a wavelength of 405 nm.

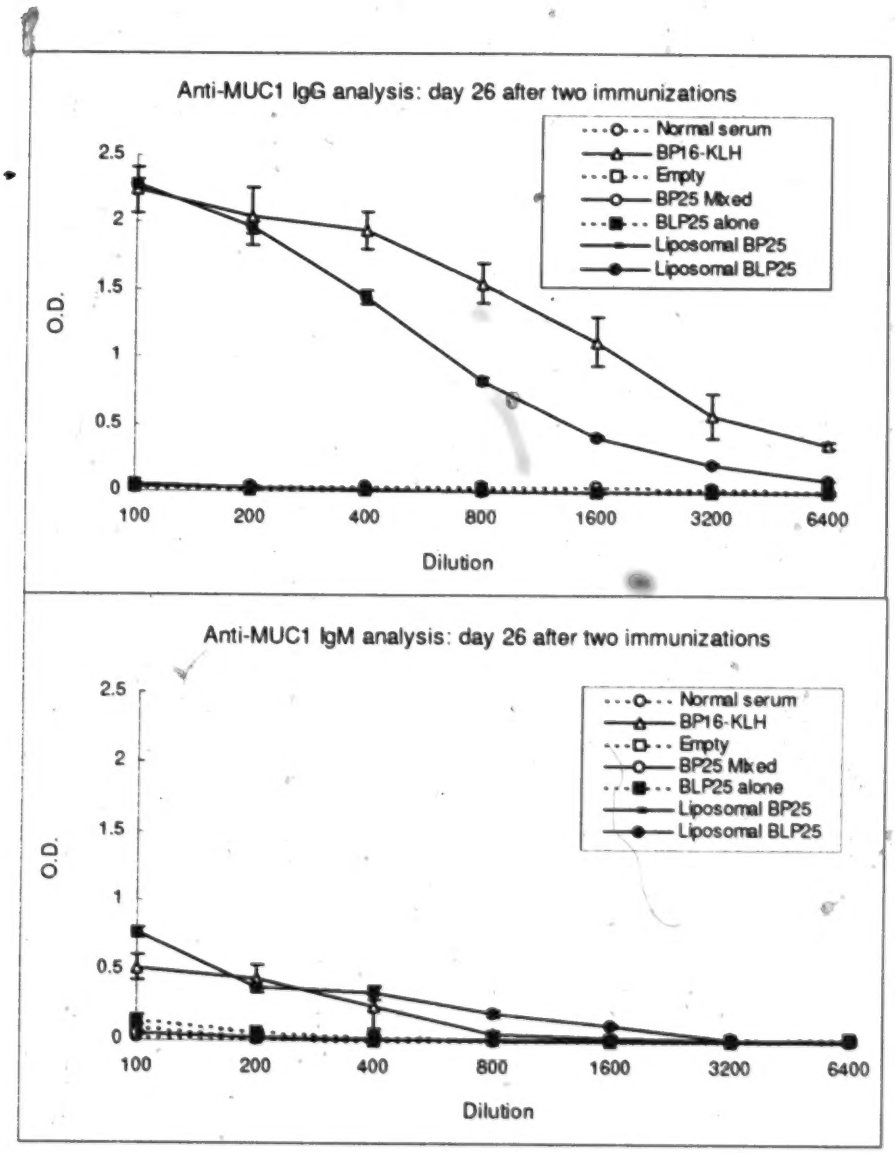

Figure 31. Anti-MUC1 IgG and IgM analyses from sera pooled from each group of the C57BL/6 mice immunized (s.c. and i.p., 14 days apart) with the five BP25 or BLP25 peptide formulations as shown. Polyclonal antiserum from BP16-KLH immunized mice served as the positive control. Optical density (O.D.) was read at a wavelength of 405 nm.

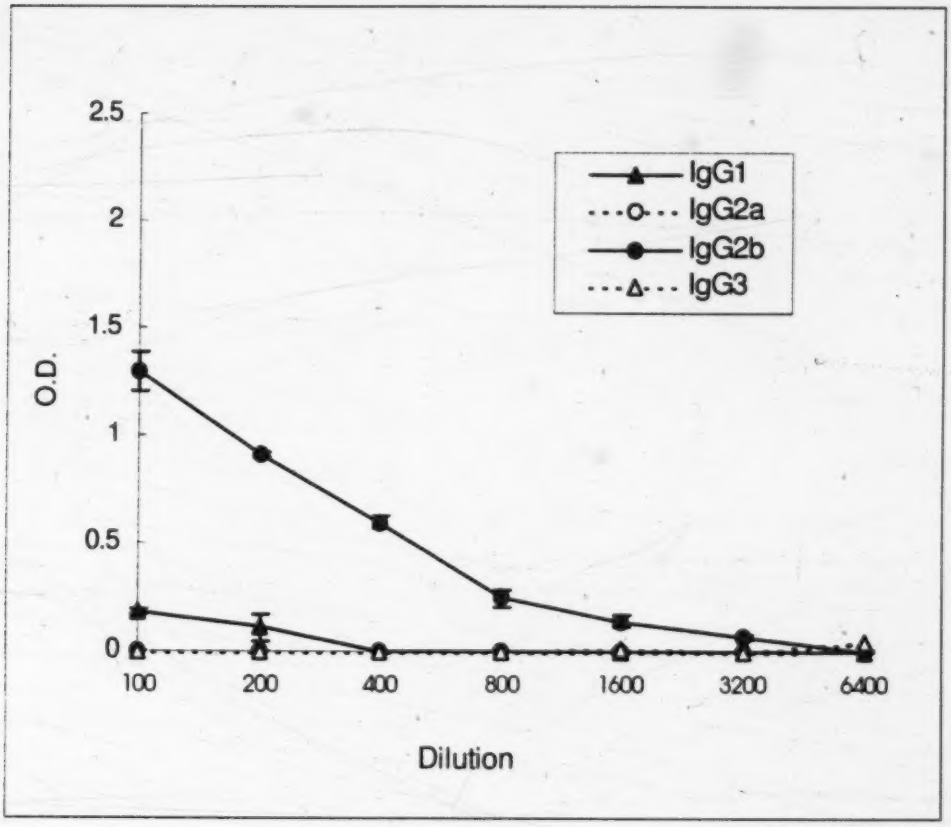

Figure 32. Anti-MUC1 IgG isotype analyses from sera pooled from the group of C57BL/6 mice immunized with the 'liposomal BLP25' preparation after two immunizations. Optical density (O.D.) was read at a wavelength of 405 nm.

b. In BALB/c Mice

MUC1 specific humoral immune responses in groups of BALB/c mice immunized with the five BP25 or BLP25 formulations as shown in Figure 32 were evaluated by ELISA using BP16-HSA as the solid phase. The positive control serum for IgG and IgM was from mice immunized with BP16-KLH.

After two immunizations, there was no significant level of IgG. However, there was IgM (approximately 50% of the positive IgM level) present in the pooled sera from the group of mice immunized with 'liposomal BLP25' formulations (Figure 33).

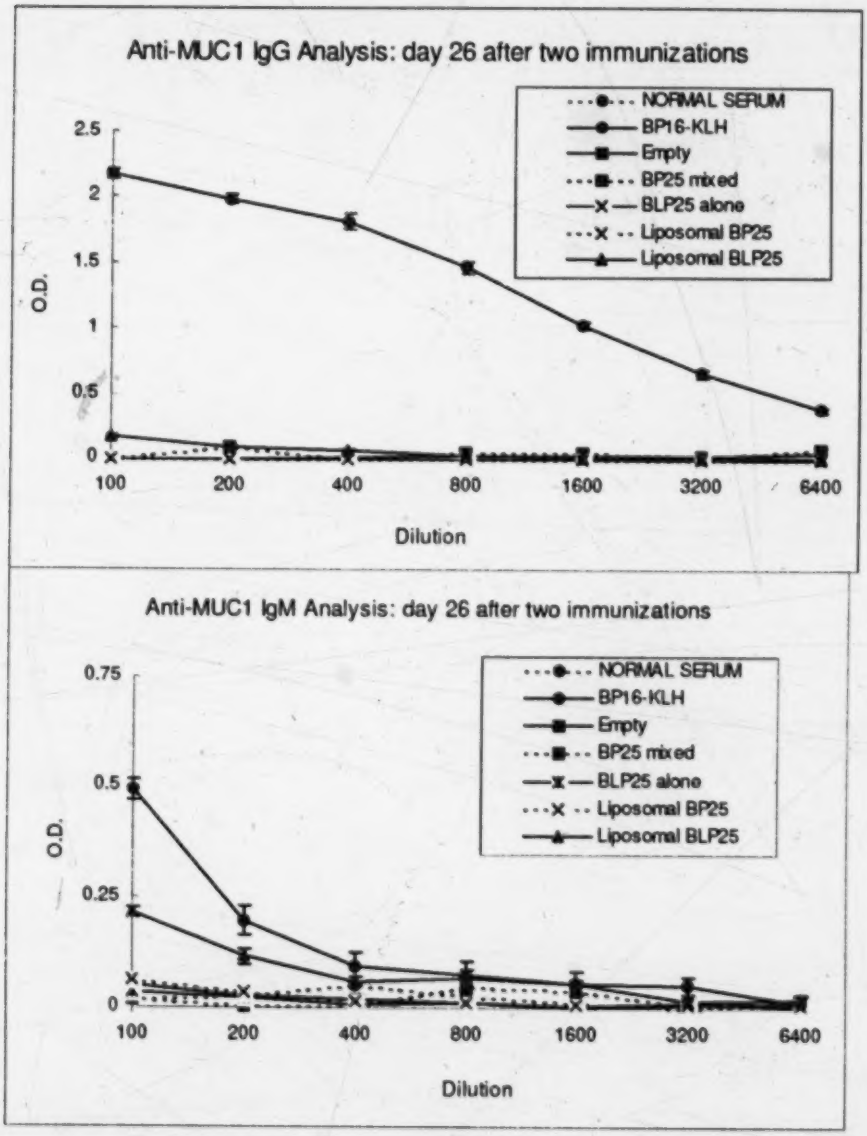

Figure 33. Anti-MUC1 IgG and IgM analyses from sera pooled from each group of BALB/c mice immunized (s.c. and i.p., 14 days apart) with the five BP25 or BLP25 peptide formulations as shown. Polyclonal antiserum from BP16-KLH immunized mice served as the positive control. Optical density (O.D.) was read at a wavelength of 405 nm.

3. Cytokine Analysis

T cell responses of human MUC1 liposomes in C57BL/6 mice were further characterized by analyzing the cytokine secretion profiles in the T cell cultures. IL-4 levels as a function of T cell culture supernatant collection time were studied. C57BL/6 mice were immunized once or twice with 'liposomal BP25' or 'liposomal BLP25' formulations. T cells ($2.5x10^5$ / well) purified from the draining lymph nodes on day 10 or day 26 were cultured along with antigen presenting cells in the presence of a human MUC1 peptide BP25. Cytokine results showed that no evidence of IL-4 was present (below 52 pg / ml) at 24 h, 48 h or 72 h in any of the T cell cultures.

However, significant levels of IFN-γ were found to be present in the T cell cultures from groups of mice immunized once with both 'liposomal BP25' and 'liposomal BLP25' (Figure 34). These results corresponded to the T cell proliferating responses (Figure 28) and also showed that the peptide location in liposomes, either masked or exposed ('liposomal BP25' or 'liposomal BLP25'), did not affect the cytokine secretion profiles after single immunization.

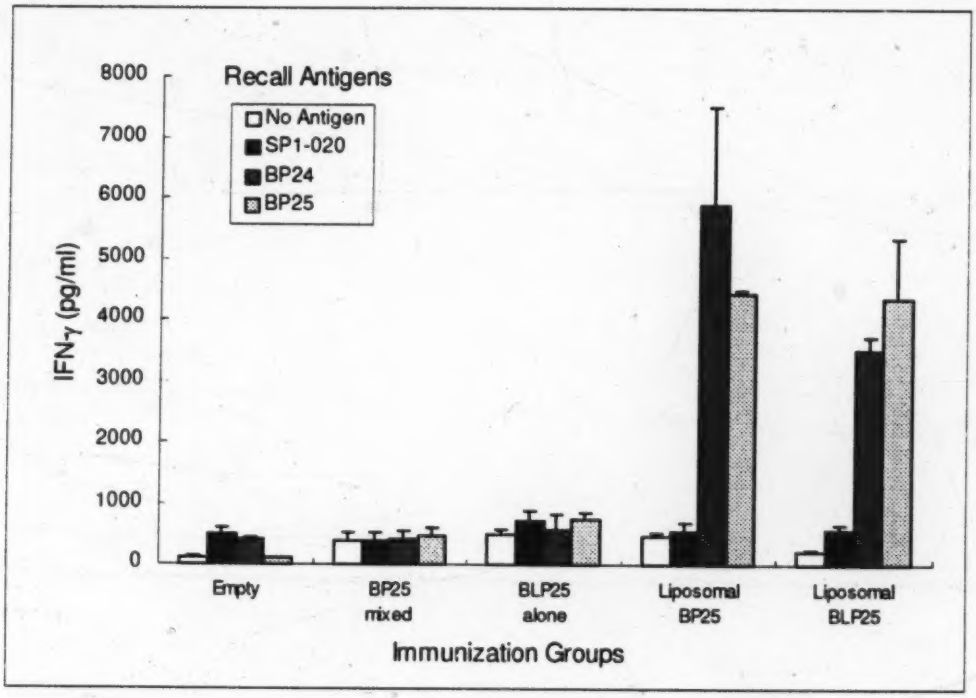

Figure 34. Levels of IFN-γ in the 72 h T cell cultures from groups of C57BL/6 mice immunized once with the five BP25 or BLP25 peptide formulations (as shown in the X axis). T cells ($2.5x10^5$/well) purified from the popliteal lymph nodes on day 10 were cultured along with antigen presenting cells alone or in the presence of human MUC1 peptides BP24 and BP25 or an irrelevant mouse MUC1 peptide SP1-020, as shown.

The above observation was also true for mice given two immunizations. High levels of IFN-γ were present in both T cell cultures from groups of mice immunized twice with either masked 'liposomal BP25' (Figure 35) or exposed 'liposomal BLP25' (Figure 36). The highest

amount of IFN-γ in the supernatant was found at the 72 h (Figure 35 and 36). These results agreed with those of given single immunization (Figure 34).

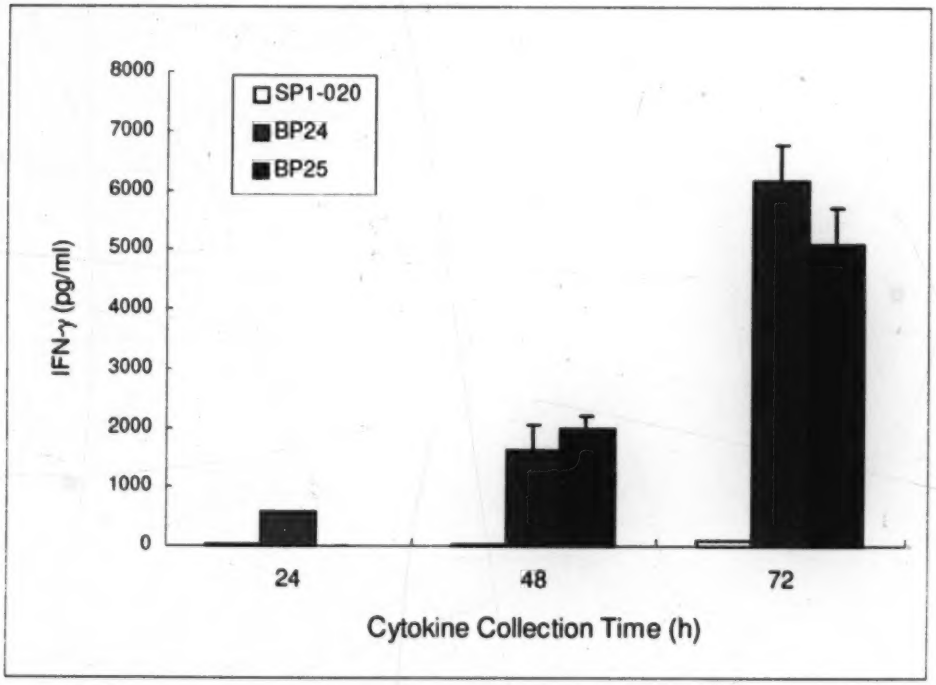

Figure 35. IFN-γ levels as a function of T cell culture supernatant collection time. C57BL/6 mice were immunized twice with the encapsulated peptide formulation ('liposomal BP25') . T cells ($2.5x10^5$/well) purified from the inguinal lymph nodes on day 26 were cultured along with antigen presenting cells in the presence of human MUC1 peptides BP24 and BP25 or an irrelevant mouse MUC1 peptide SP1-020, as shown.

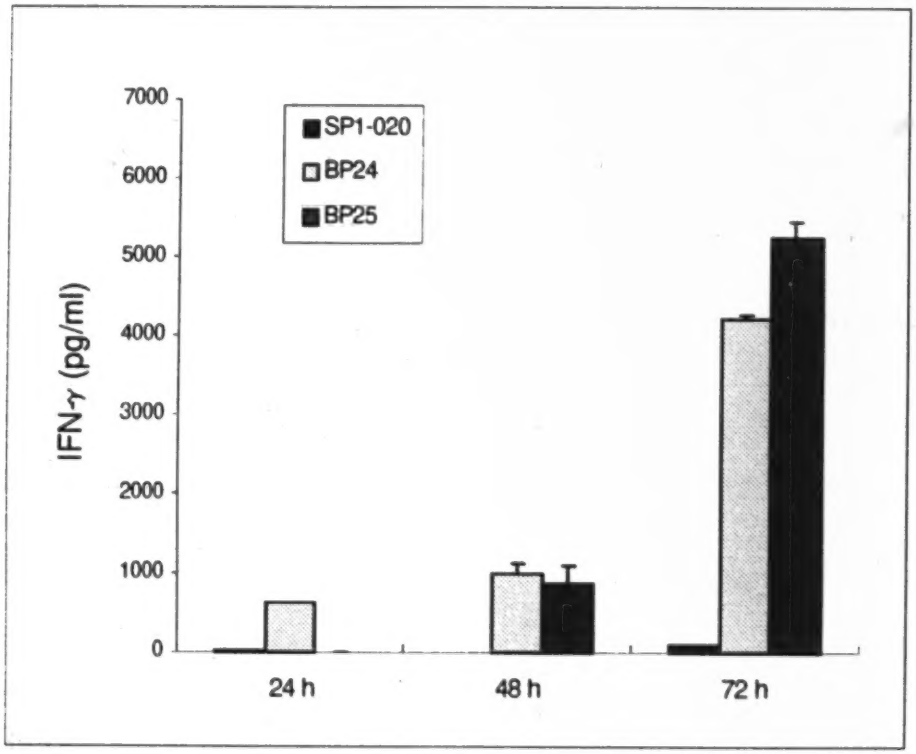

Figure 36. IFN-γ levels as a function of T cell culture supernatant collection time. C57BL/6 mice were immunized twice with the surface exposed peptide formulation ('liposomal BLP25'). T cells ($2.5x10^5$/well) purified from the inguinal lymph nodes on day 26 were cultured along with antigen presenting cells in the presence of human MUC1 peptides BP24 and BP25 or an irrelevant mouse MUC1 peptide SP1-020, as shown.

D. Effects of Peptide Dose, Liposome Composition and Size on the Magnitude of T Cell Responses

In order to assess the effects of formulation parameters (phospholipid composition and liposome size) on T cell responses, it is necessary to set up a proper evaluation system on the basis of peptide dose-dependent T cell responses. Such an evaluation model would provide an overall picture of the relationship between the degree of T cell responses and peptide doses. Revealing such a dose-dependent relationship would facilitate choosing a proper peptide dose for the assessment experiments. Thus, peptide dose ranges that would result in T cell non-responses or plateau maximum T cell responses could be avoided.

1. Peptide Dose

A peptide dose-dependent T cell response model was established for assessment of the effects of phospholipid composition and liposome size on T cell responses. A number of peptide liposomal and DETOX emulsion (as a control) formulations were evaluated in various strains of mice.

a. liposomal formulations

(i). human MUC1 BP24

Within the tested peptide dose ranges, human MUC1 specific T cell responses did not show dose-dependency in BALB/c or C57BL/6 mice. BALB/c mice responded weakly whereas C57BL/6 mice responded

equally strong from doses of 100 ng to 16 μg per mouse. The very low stimulation indices (about 2) indicated that the BALB/c mice were weak responders at peptide doses of 7 μg, 24 μg or 81 μg per mouse (Figure 37). In contrast, T cell responses in C57BL/6 mice were equally strong at peptide doses of 100 ng, 500 ng, 5 μg or 16 μg per mouse (Figure 38). In a previous study (unpublished) involving the characterization of T lymphocytes induced by the same MUC1 liposome preparations, it was shown that antigen-specific proliferation was induced by $CD4^+$ T cells, indicating Th cells were mainly involved in the T cell responses.

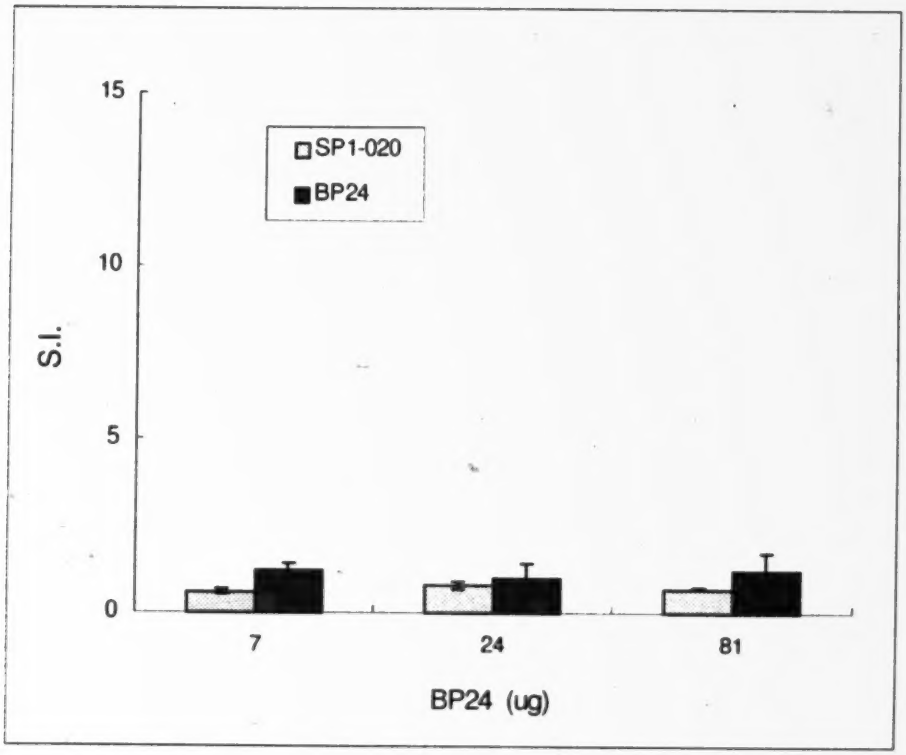

Figure 37. T cell responses of BP24 encapsulated liposomes in BALB/c mice as a function of peptide dose. T cells ($5x10^5$ / well) purified from the popliteal lymph nodes on day 10 after s.c. immunization were cultured along with antigen presenting cells alone or in the presence of BP24 or an irrelevant mouse MUC1 peptide SP1-020, as shown. Stimulation index (S.I.) was defined as the ratio of counts per min of the *in vitro* challenge peptide stimulated cultures over the peptide-free cultures.

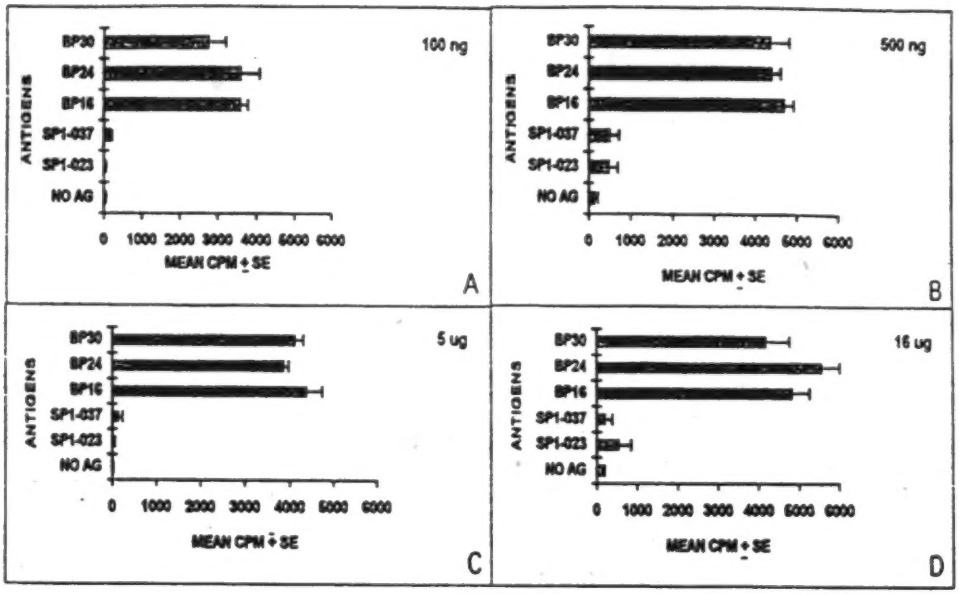

Figure 38. Human MUC1 specific T cell responses as a function of peptide dose in C57BL/6 mice. T cells ($5x10^5$ / well) purified from the inguinal lymph nodes on day 9 after the immunization were cultured along with APCs alone or in the presence of BP16, BP24, BP30, SP1-023 or SP1-037, as shown. Note: this T cell response evaluation experiment on the liposomal formulations of various BP24 peptide doses was done by Deborah Sosnowski.

(ii). mouse MUC1 SP1-020

It may be possible to show dose-dependent T cell responses by testing much larger doses of the human MUC1 peptide (>81 μg per mouse) in BALB/c mice or much smaller doses (<100 ng per mouse) in C57BL/6 mice. However, those dose ranges are not preferred from a practical point of view. Therefore, the mouse MUC1 peptide SP1-020 was evaluated in various strains of mice. SP1-020 is a consensus sequence found in mouse MUC1 molecules which have degenerate tandem repeats.

This was the first demonstration of homologous T cell responses induced by a mouse MUC1 in mice. A proper protocol for SP1-020 specific T cell responses was assessed in mice (Appendix Figure C-1). Preliminary results in C57BL/6 mice showed that there was no significant difference in T cell responses at the tested SP1-020 doses. Stimulation indexes were 10.8 and 9.6 from the immunizations with 5 μg and 35 μg SP1-020 per mouse, respectively ($P>0.01$) (Figure 39). Mouse MUC1 specific T cell responses were demonstrated in C57BL/6 mice, as evidenced by the insignificant stimulation indexes (less than 3.4) to an irrelevant recall antigen, the human MUC1 peptide BP24 (Figure 39).

Alternatively, antigen specific T cell responses were found peptide dose-dependent in BALB/c mice where SP1-020 liposomes were administrated (Figure 40). T cell responses (CPM or S.I.) were stronger (

* and ** $P<0.01$) at peptide doses of 30 μg, 60 μg or 100 μg than at 12 μg or 21 μg per mouse. There was no difference in T cell responses at doses of 60 μg and 100 μg ($P>0.01$). However, T cell responses both at 60 μg and 100 μg were stronger than that at 30 μg per mouse (** $P<0.01$).

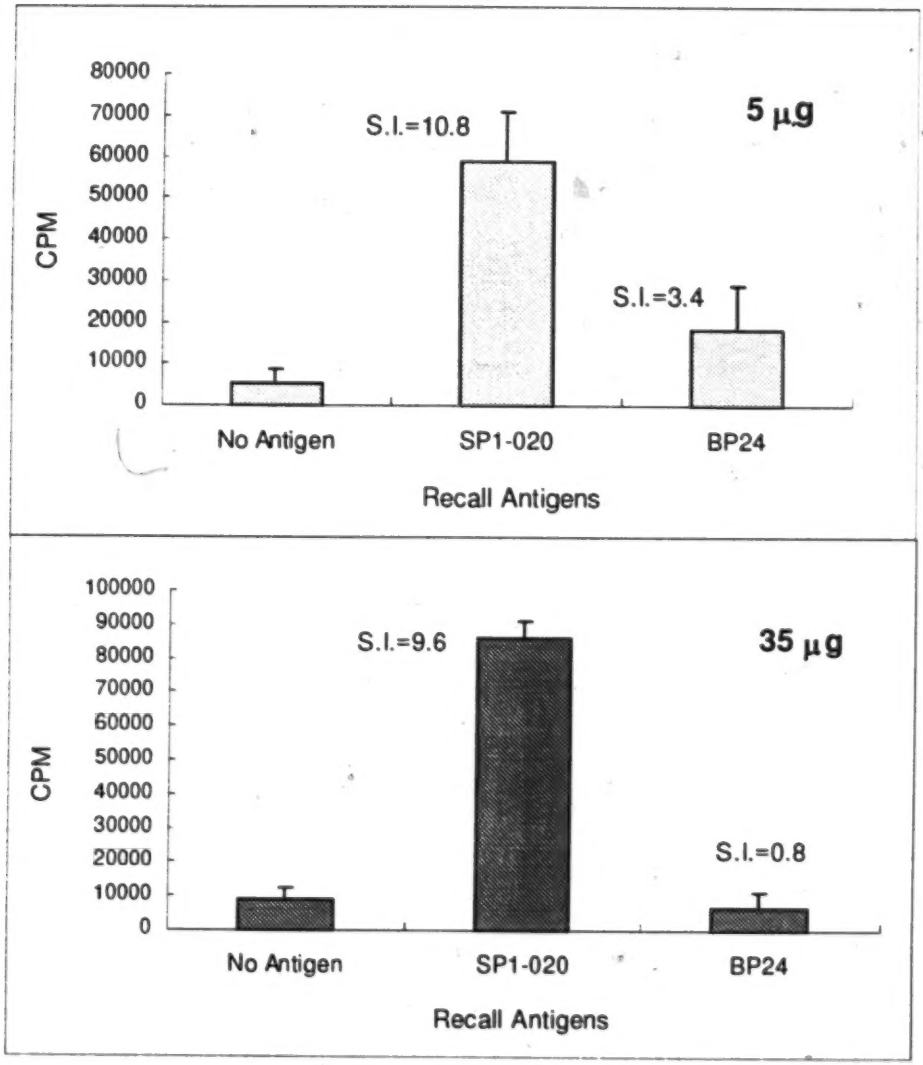

Figure 39. Mouse MUC1 SP1-020 specific T cell responses as a function of peptide dose (5 µg and 35 µg) in C57BL/6 mice. T cells ($5x10^5$/well) purified from the popliteal lymph nodes on day 10 after immunization were cultured along with APCs alone (no antigen) or in the presence of SP1-020 or BP24, as shown.

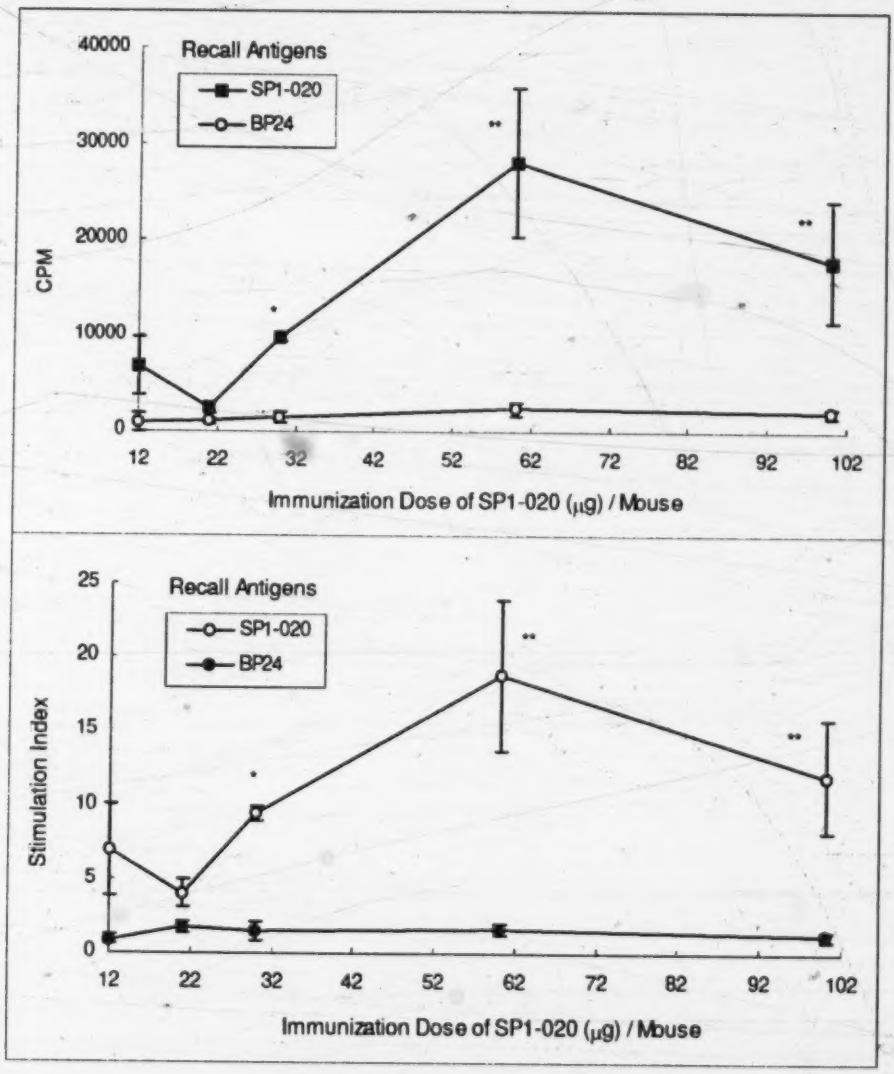

Figure 40. SP1-020 specific T cell responses as a function of peptide dose in BALB/c mice. T cells ($5x10^5$/well) purified from the popliteal lymph nodes on day 10 after immunization were cultured along with APCs alone or in the presence of SP1-020 or BP24, as shown.

*: $P<0.01$ compared to 12 μg or 21 μg.

**: $P<0.01$ compared to 30 μg.

b. DETOX adjuvant formulations

T cell responses in BALB/c mice to liposome encapsulated SP1-020 were peptide dose-dependent as shown above. However, non-specific and weak (S.I. less than 5) T cell responses were observed in BALB/c mice immunized with the DETOX adjuvant formulation for SP1-020 at peptide doses of 5 μg, 50 μg or 250 μg (equivalent to 2.2 nmole, 22 nmole or 110 nmole) per mouse (Figure 41). Previous data (unpublished) have also shown that human MUC1 BP24 DETOX formulation did not elicit any T cell responses in C57BL/6 mice, even though BP24 encapsulated liposomes stimulated strong and specific T cell responses at a dose as low as 100 ng per mouse.

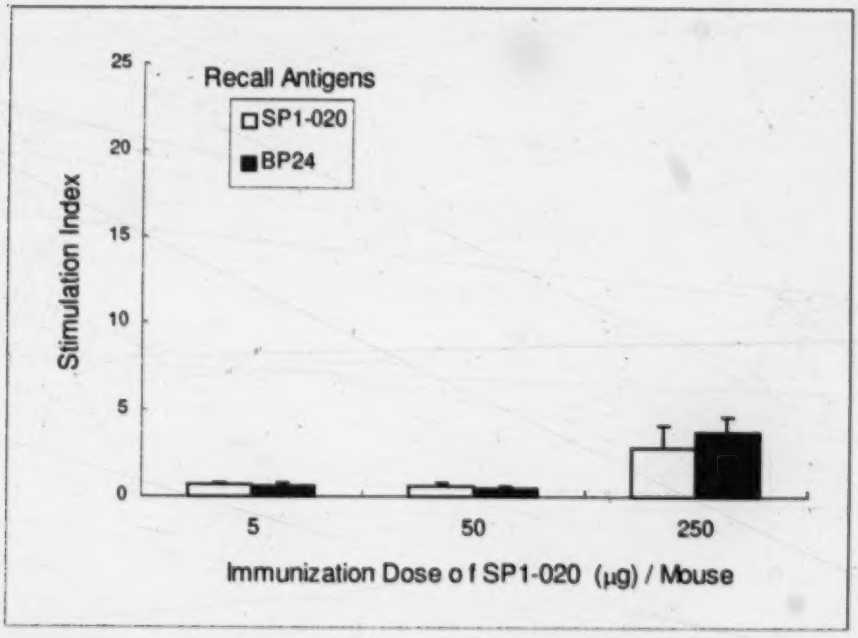

Figure 41. T cell responses induced by SP1-020 peptide formulated with the DETOX adjuvant (an o/w emulsion) at peptide doses of 5 μg, 50 μg or 250 μg per BALB/c mouse. T cells purified from lymph nodes were cultured along with APCs alone or in the presence of SP1-020 or an irrelevant human MUC1 peptide BP24, as shown. Stimulation index was defined as the ratio of counts per min of the *in vitro* challenge peptide stimulated cultures over the peptide-free cultures.

2. Liposome Composition

T cell responses (S.I. and CPM) were elicited equally strong in BALB/c mice ($P>0.01$) with SP1-020 liposomes of various phospholipid compositions: DSPC or DPPC or DMPC:CHOL:DMPG, 3:1:0.25 mole ratio (Figure 42). The SP1-020 peptide was kept at an average dose of 32 μg per mouse which was within the sub-optimal dosage range (Figure 40).

3. Liposome Size

There was no significant difference in T cell response ($P>0.01$) among various sizes of DSPC:CHOL:DMPG (3:1:0.25 mole ratio) liposome-encapsulated SP1-020 at a peptide dose of either 22 μg in liposomes prepared by the sonication method or 50 μg in liposomes prepared by the membrane extrusion method, per BALB/c mouse (Figure 43). Mouse MUC1 specific T cell responses were further characterized for cytokine secretion patterns. Results showed that there was very little IL-4 (below the detection level of 52 pg/ml) in the T cell cultures at 24 h, 48 h or 72 h whereas there were significant amounts of IFN-γ present in the cultures at 72 h (Table V).

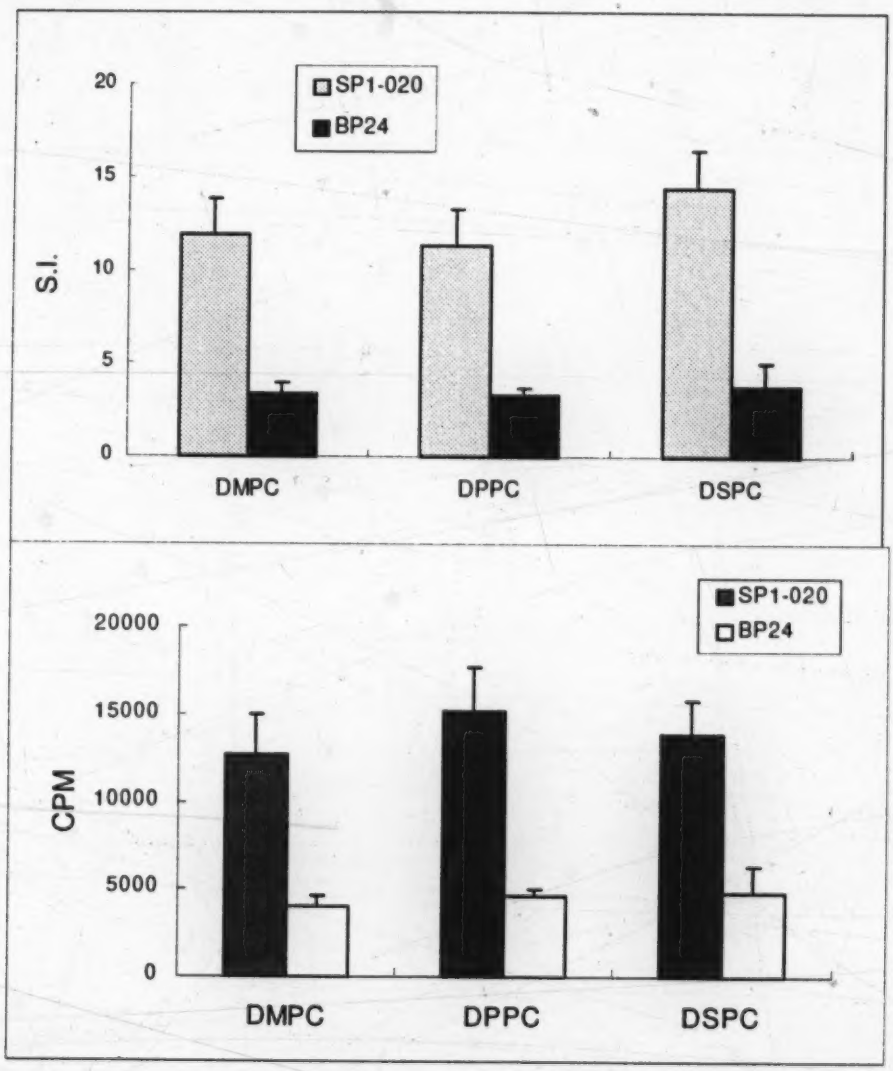

Figure 42. T cell responses induced by various phospholipid liposomes (DMPC or DPPC or DSPC:CHOL:DMPG, 3:1:0.25 mole ratio) at a peptide dose of 32 μg SP1-020 per BALB/c mouse. T cells ($5x10^5$/well) purified from the popliteal lymph nodes on day 10 after s.c. immunization were cultured along with APCs alone or in the presence of SP1-020 or an irrelevant human MUC1 peptide BP24, as shown. Stimulation index (S.I.) was defined as the ratio of counts per min of the *in vitro* challenge peptide stimulated over the peptide-free background cultures.

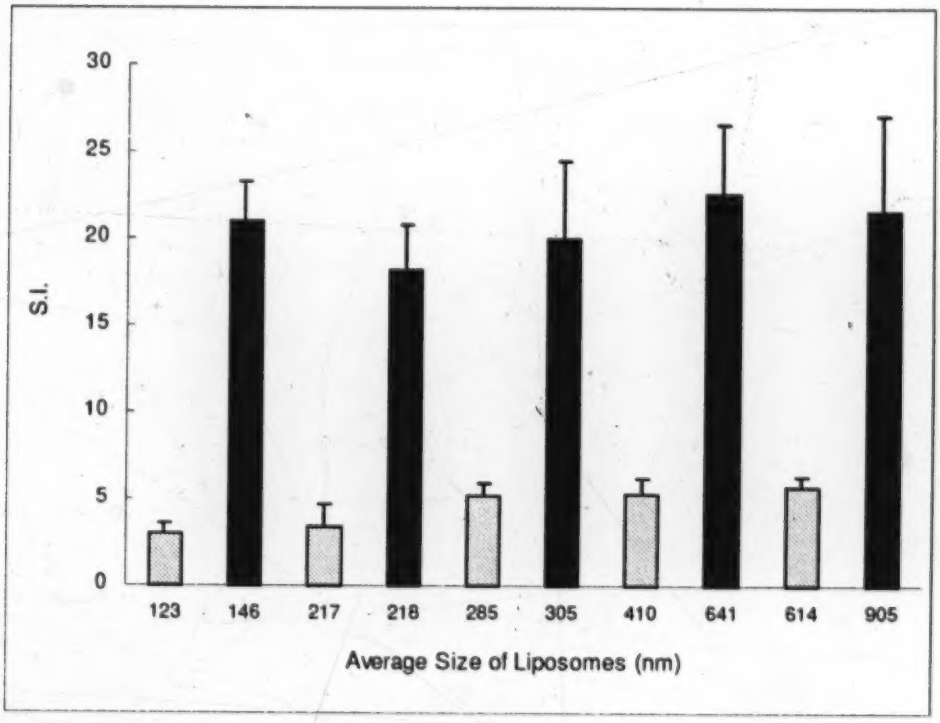

Figure 43. T cell responses induced by various sizes of liposomes at a SP1-020 peptide dose of 22 μg (light bar) or 50 μg (dark bar) per BALB/c mouse. T cells ($5x10^5$/well) purified from the popliteal or inguinal lymph nodes on day 10 after s.c. immunization were cultured along with APCs alone or in the presence of SP1-020. Stimulation index (S.I.) was defined as the ratio of counts per min of the *in vitro* challenge peptide stimulated cultures over the peptide-free cultures.

Table V. Levels of IFN-γ (pg / ml) as a function of liposome size. Cytokines were collected at 72 h after incubation of T cell cultures (BALB/c mice immunized with SP1-020 liposomes at a dose of 50 μg per mouse) in the presence of challenge peptide SP1-020 or an irrelevant human MUC1 BP24.

Size (nm)	SP1-020	BP24
146	4540 ± 150	ND*
218	4056 ± 609	ND
305	4795 ± 270	ND
641	4885 ± 250	ND
905	4300 ± 450	ND

*ND: not detectable

IV. DISCUSSION

Synthetic MUC1 peptides are potential targets for active specific immunotherapy of adenocarcinoma in humans. However, like most of the synthetic peptides, MUC1 peptides by themselves are poor immunogens (76). Liposomes offer an efficient approach to overcome the low immunogenicity associated with synthetic peptides (79,80,81). The flexibility of liposomes with variable characteristics such as size, composition, surface charge and mode of antigen association suggests versatility in vaccine design.

Th cells are the central regulating components of the immune system via cytokine secretion, and both cellular and humoral immunities rely on the Th cells to initiate and enhance immune responses (14). The fact that Th1 and Th2 cells inversely regulate growth and function of the opposing subset may lead to a reinforcement of polarizing differentiation events. Based on the particular type of a disease, a vaccine should be designed appropriately towards the protective immunity with either a Th1 or Th2 dominance. Type 1 associated immune responses have been suggested to provide effective protection against tumor and intracellular infections (47, 71, 72). The aim of this study was to develop a liposomal delivery system for synthetic MUC1 peptides favoring type 1 immune responses.

A. MUC1 Peptide Quantification

Development of methodology for MUC1 peptide quantification was a crucial step for studies of both physical characterization and immunization. The results of important formulation parameters such as encapsulation efficiency and peptide retention in liposomes depend on the reliability and reproducibility of the quantification methods. Estimation of the peptide dose administrated to mice for immune response studies also rely on an accurate method for MUC1 peptide analysis in liposomal formulations.

The traditional protein quantification Lowry method has been suggested not suitable for MUC1 peptides. Levine and Ellison reported that the Lowry method yielded only approximately one-third of known protein concentration in human saliva (120). Similar results were also obtained for purified human saliva proline-rich proteins with relatively small amounts of the aromatic amino acids (121). Smith *et al* have described an alternative assay for the quantification of proteins (116). It was found that bicinchoninic acid (BCA) is capable of forming an intense purple complex with cuprous ion (Cu^{+1}) in an alkaline environment. This finding had formed the basis of an analytical method capable of monitoring cuprous ion produced in the reaction of protein with alkaline Cu^{+2}. The color produced from this reaction was stable and proportional to protein content over a broad range of concentrations. The advantages of the BCA method are the stability of the working reagent and its greater

tolerance to commonly encountered interfering substances. Many agents, including buffers and detergents which interfered with the Lowry protein determination, were found to be compatible with the BCA assay (116).

The present study required quantification of MUC1 peptides in liposomes, so the possibility of lipid interference was studied. Results showed that presence of lipids by the BCA assay significantly interfered with MUC1 peptide quantification. This interference was found to be due to the color produced by phospholipids that reacted with the bicinchoninic acid and due to the fact that the magnitude of the combined absorbances varied with the type and quantity of phospholipid (122). This kind of variation made it difficult to simply subtract out the contribution from the lipid in the determination of peptides and proteins.

Because of its excellent resolving power and reliability, reversed phase HPLC has become a dominant method in recent years for both analytical and preparative separations of peptides and proteins (123). A C18 stationary phase and a gradient elution program with increasing concentration of the organic modifier acetonitrile in the mobile phase were used for HPLC analysis of MUC1 peptides. Phenol was used as the internal reference. This MUC1 quantification method was found reliable, reproducible and highly sensitive. Most importantly, the HPLC method was found to be suitable for direct analysis of MUC1 peptides encapsulated in liposomes.

Analysis of peptides in liposomes required solubilization of the liposomal pellets. However, the non-ionic detergent Triton X-100 showed undesirable problems during HPLC analysis, causing interference in elution profiles. Alternatively, absolute ethanol was found more suitable for pellet analysis in terms of 1) reliable elution profile with a smooth baseline and with the same running duration as peptide in PBS, 2) much clearer solution from the solubilization of liposomal pellets compared to the one by Triton X-100, and most importantly, 3) high peptide recovery as verified by two methods.

After solubilization by absolute ethanol, empty liposomal pellets were spiked with a known amount of MUC1 peptide and phenol. Excellent recovery (nearly 100%) of the peptide was obtained. Secondly, greater than 87% of the initial MUC1 peptide used for lipid film hydration was recovered by the HPLC analysis of supernatants and pellets from liposomal aliquots after incubation or storage. The recoveries (87% to 93%) seen in mass balance study indicated a satisfactory, if not complete, solubilization of liposomal pellets by absolute ethanol. These recovery results are not surprising since the effect of ethanol on liposomal bilayers has been reported (124, 125). In the presence of ethanol, a normal lipid bilayer and interdigitated structure coexist and the lipid membrane is in a phase-separated state (124). The high permeability of liposomal membrane induced by the instability of the boundary regions could account for the high recovery results (125).

B. MUC1 Peptide Liposome Formulation

Since the discovery of liposomes by Bangham and Horne in 1964, the flexibility, safety and capability for entrapping a wide range of molecules have made liposomes a very useful tool in the field of drug/ antigen delivery (82, 126). An important pre-requisite for liposome application in vaccine formulation is antigen incorporation in liposomes.

Encapsulation efficiency is mainly influenced by the hydrophilic/hydrophobic nature of the molecules of interest (84). For most lipophilic and amphiphilic molecules, high entrapment (greater than 60%) can be readily achieved in neutral, negatively- and positively-charged liposomes composed of a variety of phospholipids (83, 127, 128). In the present study, 80-85% of acylated MUC1 BLP25 (an amphiphilic peptide) was incorporated in liposomes. However, the problem of low entrapment of hydrophilic MUC1 peptides required a solution. A working hypothesis is that encapsulation efficiency of hydrophilic molecules is a function of the size of aqueous compartments in liposomes when electrostatic binding with lipid bilayers is insignificant (84, 85). The human and mouse MUC1 peptides used in the present study were highly water-soluble molecules.

Results have indicated that addition of a charged lipid component (DMPG or SA) to the bilayers significantly increased the encapsulation efficiency of BP24 or SP1-020 in liposomes of various phospholipid compositions (DMPC, DPPC or DSPC). Interbilayer separations (as

detected by X-ray analysis) can be readily increased from as little as 2.5 nm to 10 nm or more by incorporation of relatively small amounts of charged lipid components (129). Hence, the presence of charged lipids increased the electrostatic repulsion between bilayers resulting in enlargement of the aqueous compartments in liposomes and, consequently, much higher encapsulation efficiencies (127, 130). This was the case in the improved BP24 encapsulation efficiency.

The encapsulation efficiency was not influenced by pH from 4-7.4, indicating that peptide association with the lipid bilayer as a result of electrostatic interaction was insignificant. In contrast, the encapsulation efficiency of BP24 (pI=5.5) was significantly increased but only at pH of 2 (data not shown). It has been stated that the electrostatic association would contribute to encapsulation efficiency only when there is a sufficient difference between pH of the medium and pI of the solute molecule (131).

Higher encapsulation efficiency of a water-soluble molecule Ara-C (pKa = 9.7) was observed at pH 4.5 in FAT MLV liposomes composed of DPPC:CHOL:DCP (pKa of DCP = 4.5) than in liposomes of DMPC:CHOL:DCP (132). Also, the patterns of encapsulation efficiency as a function of DCP were different. Proportional uptake of Ara-C was observed in the DMPC liposomes but not in the DPPC liposome composition (132). An increased volume of aqueous compartments in DPPC liposomes combined with the rigid structure of the gel-state DPPC

bilayers (Tc=41°C) hindered binding of positively-charged Arc-C to negatively-charged sites in the bilayers. On the other hand, the more efficient binding of Ara-C molecules to negatively-charged DCP molecules in fluid-state bilayers presumably accounted for the proportional uptake of Ara-C in DMPC:CHOL:DCP liposomes.

In the present study, the encapsulation efficiencies of BP24 in DPPC or DSPC liposome compositions were higher than in DMPC liposome compositions, however, no proportional uptake of the MUC1 peptide was observed in the DMPC liposome compositions. On this bases, the peptide was unlikely associated with the negatively-charged lipid bilayers because at pH 7.4 BP24 (pI = 5.5) is also negatively charged (133). In this case, the space of aqueous compartments in liposomes rather than electrostatic interactions between peptide and bilayer is believed to be the dominating factor in encapsulation.

The encapsulation efficiencies of BP24 and SP1-020 were significantly improved as a result of reduction in ionic strength of the aqueous buffer (Figure 12 and 13). This may be expected since the surface charge of bilayer is reduced at high ionic strength due to screening of the charge by electrolytes in the medium (134, 135). A reduced electrical repulsion of the bilayers results in decreased volume of aqueous compartments in the liposomes and, consequently, decreased encapsulation efficiencies. Consequently, higher encapsulation efficiencies were observed at low ionic strength (129). The levels of

incorporation of hydrophilic peptides such as human MUC1 peptide BP24 or mouse MUC1 peptide SP1-020 are in the range observed with other water-soluble drug molecules (84, 85, 127, 136).

The rationale for using liposomes as antigen delivery systems for induction of immune responses is based on the known preferential uptake of liposomes by antigen-presenting cells (91, 137). In this case, a stable liposomal formulation is needed to achieve effective delivery. Ideally, liposomes should retain incorporated peptide antigens until *in vivo* administration and/or uptake by antigen-presenting cells.

Results of storage stability with greater than 90% retention of BP24 in liposomes after 31 days and greater than 85% retention after at least 12 months suggest coherently-structured and tightly-packed lipid bilayers of DMPC, DPPC or DSPC liposomes at 4°C. However, at 37°C DMPC or DSPC liposomes became leakier in serum than in PBS probably due to plasma proteins and particles which induce bilayer permeability (138, 139). The extent of release of a polar molecule from liposomes depends very much on a number of factors including temperature, composition, size and lamellarity of the liposomes (140). In the present study, DPPC liposomes did not follow the trend of DMPC or DSPC liposomes. There was no additive induction in membrane permeability observed in DPPC liposomes in mouse serum, although they were leakier than DMPC or DSPC liposomes in PBS at 37°C, likely because the incubation temperature of 37°C is in the range of bilayer structure weakness

between the pre-transition and main-transition temperatures of DPPC liposome compositions (pre-Tc =34°C and Tc = 41°C).

Liposomes used in this study contained more than 23 mole percent cholesterol and they were relatively stable (greater than 75- 80% peptide retention) during incubation with 100% mouse or human serum up to 24 h. This is consistent with the understanding that such liposomes are less susceptible to serum-induced release of entrapped molecules (138, 141). It has been found that inclusion of a certain amount of cholesterol into the liposome structure can significantly enhance liposome stability by preventing phospholipid loss as a result of interactions between liposomes and serum components, such as high-density lipoproteins (HDL). At less than 20 mole percent cholesterol little effect on liposome stability is observed whereas more than 20 mole percent cholesterol enhances liposome stability dramatically (141).

C. Nature of Peptide Association with Liposomes on the Type and Magnitude of Immune Responses

Liposomes may exert their adjuvanticity as 1) a slow release "depot" of antigen at the site of injection similar to many other particulate adjuvants, 2) a "protector" for encapsulated proteins or peptides from enzymatic degradation, 3) a "vehicle" for efficient delivery of associated antigens to APCs and, consequently, enhance immunogenicity of the antigen (95, 142-144). It was found that a much lower concentration of

liposomal-associated antigens than non-associated antigens was required for T cell proliferation response (97). Consistent with the literature, both single and double immunizations showed that peptide association with liposomes was required for induction of MUC1 specific T cell response at a peptide dose of 2 nmole per mouse. Strong and specific T cell responses were elicited by liposome-associated MUC1 peptides, either encapsulated or surface-exposed, suggesting efficient delivery of the peptides to APCs whereas non-associated peptides, either peptide alone or mixed with 'empty' liposomes, failed to induce strong T cell responses, probably due to either rapid enzymatic degradation or not being able to be efficiently delivered to APCs.

Most of the peptides have a history of fast degradation in serum, i.e., with a half-life of several minutes (145, 146). Thus, lack of protection from peptidases by liposomes would be expected to be one of the reasons for non-immunogenicity of the non-liposome-associated 'free' MUC1 peptides. For most protein antigens, potent immune responses may be induced with a variety of liposomal formulations, where protein antigens are entrapped, surface-linked, or even mixed with liposomes (106, 147, 148). Protein antigens containing hydrophobic domains may physically associate with lipid bilayers simply by mixing with liposomes. However, hydrophilic MUC1 peptides used in this study are unlikely to associate with lipid bilayers, suggesting that MUC1 peptides simply mixed with liposomes are not taken up by the APCs as efficiently as liposome-

associated peptides. It may be concluded that liposomes exert their adjuvant effects by serving as a vehicle for the associated MUC1 peptides and immunomodulator MPLA to the APCs as well as offering protection of the peptide from enzymatic degradation.

The proposed mechanisms of MUC1 liposomal adjuvanticity were also indicated by the comparison study of DETOX MUC1 formulations in mice. DETOX is an oil-in-water emulsion and the adjuvanticity is due to its ability to adsorb antigens at the surfaces of oil droplets which are preferentially taken up by antigen-presenting cells. However, hydrophilic MUC1 peptides used in this study are not likely associated with the oil droplets to any significant degree. Consequently, weak and non-specific T cell responses were induced by the DETOX preparations, even at very high peptide doses (50 and 250 μg SP1-020 per mouse). Therefore, the o/w emulsion may not be suitable as an effective adjuvant for hydrophilic antigens such as MUC1 peptides.

A stable liposomal formulation is required to serve as an effective MUC1 delivery vehicle. The integrity of MUC1 FAT MLVs incubated with 100% serum was evident for at least 24 h as shown by liposomal size determinations. Retention of more than 75% MUC1 peptides by liposomes incubated with 100% serum up to 24 h suggested that the majority of the peptide was delivered to the immune system in an associated rather than 'free' form. It has been demonstrated that injected liposomes are retained in various tissues for extended periods of time

with a half-life of 10-13 h based on the percentage of liposomes remaining intact (149). Liposomal markers were found accumulated mainly in the liver and spleen, and intact liposomes with a half-life of 12 h appeared to be taken up by these organs (150). Stability of MUC1 liposomes is supported by *ex vivo* results of strong and specific T cell responses even at very low peptide doses (Figure 28 and 29).

It has been previously proposed that the topographical location of antigen in liposomes may affect the type of T cell response. Based on the immunoglobulin isotype profiles, it was shown that liposomal surface-exposed antigen preferentially followed a Th1 whereas encapsulated antigen followed a Th2 activation pathway (110). However, as demonstrated in the present study by either single or double immunizations, the type of T cell responses was independent of the nature of MUC1 association with liposomes. The cytokine secretion pattern of high levels of IFN-γ and no evidence of IL-4 indicated that type 1 immune responses were elicited by either encapsulated or surface-exposed MUC1 liposomes (15). These results are consistent with a recent study that, based on cytokine profiles, showed location of antigen in liposomes had no effect on the type of T cell responses (106).

The exact mechanisms by which these liposomal formulations favor immune responses of the Th1 type have not been examined. It is hypothesized that MUC1 delivery by these MPLA-containing liposomes may result in IL-12 production, a key cytokine initiator of the type 1

immune responses (19, 151, 152). For example, it was demonstrated that T cell lines generated in the presence of IL-12 exhibited a reduced ability to produce IL-4 and an increased ability for IFN–γ production (152).

It has been proposed that liposomes might preferentially induce Th1 type immune responses via the activation of macrophages and dendritic cells which are considered as preferring type 1 immune responses (91, 151). Uptake of particulates such as has been reported to induce IL-12 secretion by macrophages and dendritic cells (151).

Unlike traditional adjuvants such as mineral oil emulsions or alum that constitute depots at injection sites, the proposed mechanism using lipid A is by inducing local macrophages to synthesize IL-12 which activates natural killer cells to consequently produce IFN–γ (19, 153, 154). Local IFN–γ next primes macrophages to process antigen and to serve as APCs. The production of a high local concentration of IFN–γ by natural killer cells leads to selection of the type 1 immune responses.

Investigation of the mechanism of adjuvant action of liposomes containing lipid A has led to two interesting proposed mechanisms (111). First, this formulation caused an intensive recruitment of macrophages that presumably have the ability to serve as APCs for liposomal antigens; and second, liposomes with lipid A caused as much as a 20-fold increase in the presentation of antigens compared to liposomes lacking lipid A, as determined by proliferation assay of an antigen specific T cell clone. Synergetic adjuvant effects have been found with liposomes containing

certain immunomodulators such as lipid A, muramyl dipeptide or its derivatives (42, 80). In combination with other immunoadjuvants, liposomes have been reported to induce very strong immune responses. The polarized type 1 immune responses might be a result of the additive adjuvant effects of liposomes and MPLA.

The first report of liposomal adjuvanticity described that liposome-encapsulated protein antigens induced up to a 10-fold antibody response compared to free antigens (79). However, the effect of topographical location of antigen in liposomes on humoral immune response has been controversial. It was deduced that liposomal encapsulation did not enhance immunogenicity of antigens (102, 103, 155). In the early 1980s, van Rooijen *et al* found that although the amount of antigen entrapped in liposomes was much higher than surface-coated, liposomes containing both entrapped and coated antigen did not elicit stronger humoral immune response than 'empty' liposomes to which antigen was only adsorbed (102). As a result, the adjuvant effect of liposomes is likely mediated by surface-exposed rather than encapsulated antigen (102, 103). In contrast, results of other studies indicated that encapsulated antigens were not only able to induce humoral immune responses but also stronger responses occurred compared to only surface-exposed antigens (104, 105). Interestingly, the response was not diminished by treatment of the preparations with trypsin to remove adsorbed protein antigens (105). The most used antigens in these studies were human

serum albumin (HSA) and bovine γ-globulin (BGG), which are non-specifically associated with liposome bilayers via hydrophobic bonding (156, 157). As a result, surface adsorption of the antigen during liposome preparation can not be avoided. This has led to controversy regarding the relative contribution of encapsulated and surface-exposed antigens to antibody production. Thus, it would be important to clearly demonstrate the role of antigen location in liposomes on immune responses, especially in the case of water-soluble antigens which may be either encapsulated or surface exposed.

Most of the studies have involved protein antigens, little work has been done with synthetic peptides. The hydrophobicity of peptide antigens also seems important for the induction of humoral immune responses. High levels of antigen-specific antibodies were generated by immunization of mice with liposomes encapsulating non-modified synthetic peptides corresponding to the 25 amino acid sequence of an enzyme (158). The primary epitope was not exposed at the surface and is most likely sequestered in a pocket-like conformation in the native enzyme. Although there is no experimental data showing peptide location in liposomes, the information that 12 out of the 25 amino-acid residues are hydrophobic can not be ignored (158).

In the present study, water-soluble MUC1 peptide BP25 was used to evaluate the nature of antigen association with liposomes on T cell (as discussed earlier) and humoral immune responses. Characterization of

the surface expression of MUC1 specific epitopes clearly showed that these epitopes were not present on liposomes encapsulated with non-modified hydrophilic MUC1 peptides BP24 or BP25 whereas they were present, as anticipated, on liposomes with N-terminal acylated peptide BLP25 (Table I). Unexpectedly, however, the MUC1 epitopes were not detected in liposome-encapsulated BLP24, a C-terminal acylated MUC1 peptide (Table I), using the same mAb B248.7R2 as in BLP25 liposomes. Hence, it is suggested that the coupling site of a lipid tail to the peptide (N-terminal versus C-terminal) influenced the MUC1 epitope specific for B248.7R2 following lipid bilayer insertion of the peptides acylated at different sites. Most likely, the MUC1 epitope specific for B248.7R2 assumed different conformational preferences as a result of liposomal anchoring via the lipid tail occurring at either end of the MUC1 peptide (88).

Experiments with acylated peptide CGP18 derived from the V3 loop of envelope gp120 of HIV-1 showed that the resultant orientation of the peptide in liposomal bilayers was critical for detection of relevant epitopes by specific antibodies as well as for antibody induction (36). Two mAbs specific for different epitopes of CGP18 each recognized the N-terminal acylated peptide, but neither recognized the C-terminal acylated CGP18. This observation was accompanied by corresponding parallel abilities of the liposome-associated molecules to induce antibodies to CGP18. High levels of antibodies to CGP18 were induced by liposomes encapsulated

with the N-terminal acylated peptide, but not with the C-terminal acylated peptide (36). By performing computerized molecular modeling, it has been shown that different conformational preferences resulted from each of the acylated CGP18 molecules following lipid bilayer insertion. It is suggested that the steric hindrance at the liposomal surface might have prevented access of the peptides by cellular elements of the immune system responsible for antibody production (88).

The evidence that there was no MUC1 specific antibody induction by BP25 liposomes at a peptide dose of 2 nmole per mouse is consistent with previous data showing no anti-MUC1 antibodies detectable in mice immunized with BP24 peptide liposomes. However, this study also demonstrated that surface-exposed BLP25 formulations induced high levels of anti-MUC1 antibodies, suggesting that surface exposure of MUC1 epitope is required for antibody induction. Although BLP24 liposomes were only analyzed for surface exposure of the MUC1 epitopes and not assessed for antibody induction, immunization of mice with another C-terminal acylated MUC1 peptide (BLP25-C-terminal acylation) encapsulated liposomes induced MUC1 specific antibodies (Biomira, unpublished data). It was an indication that steric hindrance at the liposomal surface might not be a significant factor for MUC1 peptides. These results, however, further supported the hypothesis that the lipid tail coupling site of the MUC1 peptides might have influenced conformational

preferences of the MUC1 epitopes following lipid bilayer insertion, at least in the case of B248.7R2 specific MUC1 epitopes.

Both masked and surface-exposed peptide liposomes would be expected to be phagocytosed and processed by professional APCs such as dendritic cells and macrophages. However, only liposomes with surface-exposed peptide would be expected to interact with B cells. Dal Monte and Szoka demonstrated, via an *in vitro* antigen presentation system, that soluble and liposome surface-attached, but not encapsulated antigen was processed and presented by B cells (159) whereas the encapsulated antigen was phagocytosed and presented by macrophages (160). B lymphocytes can only be activated by recognition of peptides at liposome surfaces via antigen-specific Ig receptors, leading to peptide specific antibody production (161, 162). The lack of antibody production by BP25 encapsulated liposomes is probably due to the lack of antigen accessibility to the Ig molecules on B lymphocytes. On the other hand, induction of strong and specific MUC1 IgG antibodies by BLP25 liposomes may be attributed to the accessibility of MUC1 epitope to B cells. Results of this study support the hypothesis that liposomes differing in antigen location may preferentially activate different immune pathways, namely, cellular and humoral, by activating different groups of antigen presenting cells (163).

To further assess T cell dependency of anti-MUC1 antibody production, various human MUC1 liposomal formulations were evaluated

in a weak-responder strain of mice. The result was that insignificant T cell response (stimulation index <2) was induced in BALB/c mice by human MUC1 liposomal formulations at the tested doses. When BALB/c mice were immunized twice with BP25 or BLP25 liposomes, there were no MUC1 specific IgG antibodies found in mouse sera, the exception being some anti-MUC1 IgM antibodies by BLP25 liposomes, indicating T cell-dependent MUC1 specific humoral immune responses.

MUC1 specific antibodies induced by liposomal BLP25 were dominated by IgG2b over IgG1 with no detectable levels of IgG2a or IgG3. The distribution of Ig isotypes may be influenced by many factors, including antigen location, mouse strain, adjuvants and the route of administration. The following are several examples. Using liposomal surface-conjugated peptides, predominant production of IgG2b over IgG1 was also observed (164). The ratio of IgG2a / IgG2b has been found to be influenced by the strain of mice, with a predominance of IgG2a in A/J mice and a predominance of IgG2b in C57BL/6 mice (165, 166). Immunomodulatory properties of adjuvants can also affect Ig isotype distribution. Enhanced expression of IgG2b over IgG1 was observed in lipid-soluble MDP (muramyl dipeptide) liposomes but not in water-soluble MDP liposomes (167). It has been reported that lipid A and its analogous immunomodulators prefer type 1 associated immune responses including certain antibody isotypes such as IgG2a and/or IgG2b production in mice (168, 169). For example, it was observed that mice immunized with an

influenza vaccine containing MPLA produced a 5-fold increase of both IgG2a and IgG2b titers, and a 3-fold decrease in IgG1 titer (168).

The presence of IgG isotypes have been considered as a measure of the protective quality of the immune response to a specific antigen. In the present study, anti-MUC1 IgG2b dominated antibody production was accompanied with high levels of IFN-γ but no evidence of IL-4, suggesting type 1 associated antibody responses. It will be interesting to see whether those anti-MUC1 IgG2b antibodies would be protective from MUC1 expressing tumors or not in the future.

The results of strong and specific type 1 immune responses induced by the MUC1 liposomal formulations in the present study are consistent with a recent study of our group (170). With BP24 encapsulated liposomes containing 1% MPLA, protection from human MUC1 transfected murine mammary adenocarcinoma correlated with MUC1 specific T cell proliferation, IFN-γ and some anti-MUC1 IgG2a antibodies; whereas lack of protection correlated with MUC1 specific IgG1 with low or no IgG2a antibodies and low or no MUC1 specific Th1 responses in CB6F1 (BALB/c X C57BL/6) mice. There was no anti-MUC1 antibodies in mouse serum after immunization with BP24 liposomes and MUC1 specific IgG2a or IgG1 antibodies were only found after challenge with a human MUC1 expressing murine tumor cell line (170). Consistent with the present study, surface-expressed MUC1 epitopes on tumor cells might account for antibody induction. The protection against MUC1

expressing tumors may be attributed to the type 1 associated immune responses as evidenced by the presence of MUC1 specific IgG2a accompanied with IFN-γ in the protected mice. However, the exact mechanisms regarding roles of humoral and cellular immune responses in providing effective protections are still not clear yet as discussed in the introduction.

D. Role of Phospholipid Composition and Liposome Size on T Cell Responses

The magnitude of T cell responses may be influenced by many factors, including the nature and form of the antigen, dose and route of administration of the antigen and the genetic background of the individual (4). The ability of a given antigenic determinant to associate with the MHC molecules of a particular individual would determine the degree of T cell responses. In BALB/c mice, a weak responder, human MUC1 BP24 peptide did not stimulate a strong T cell response even at 81μg, however, in C57BL/6 mice, a strong responder, a dose of 100 ng induced a strong T cell response. These results exemplify the influence of genetic constitution and immunodominance on the magnitude of a T cell response (7, 171).

Although a dose-dependent T cell response may be observed by using human MUC1 peptides at very high doses in BALB/c mice or extremely low doses in C57BL/6 mice, practically the preferred dose must

take into account peptide cost as well as reproducibility and reliability of immunization results. For the first time, a strong and specific homologous T cell response induced by a consensus mouse MUC1 peptide has been demonstrated. In BALB/c mice, such an homologous T cell response was peptide dose-dependent within the practical dose range. Consequently, the evaluation system of SP1-020 in BALB/c mice and a 'sub-optimal' peptide dose region were chosen for the assessment studies on phospholipid composition and liposome size.

It has been suggested that phospholipids might affect the binding of peptide to MHC molecules by directly or indirectly inducing conformational changes in class II molecules that favor their peptide-binding property (112). It was found that some phospholipids (i.e. phosphatidylcholine) enhanced the binding 10- to 50-fold, whereas others (i.e. phosphatidylethanolamine) had no effect. Any increase in peptide binding was also dependent on the length of phospholipid fatty acyl chain, for example, myristoyl, palmitoyl or stearoyl group but not caproyl or lauroyl chains (112). The extent of binding of peptide to MHC molecules also usually correlates with their immunogenicity via T cell responses, suggesting that the magnitude of a T cell response may be influenced by the phospholipid composition (7, 112, 172).

There were no significant differences in T cell responses among liposomes composed of various phospholipids (DMPC, DPPC or DSPC), indicating chain length independence of binding of peptide to MHC

molecules (112). An immunization dose at the 'sub-optimal' dose level was maintained for three of the liposomal formulations and it had been shown that these liposomes were stable in 100% serum, suggesting comparable delivery of peptide to antigen-presenting cells resulting from similar levels of peptide presentation to T cells both quantitatively and qualitatively. Although other factors involved in the process of T cell proliferation can not be excluded, the result is not surprising.

By applying the liver perfusion technique, two typical studies have demonstrated that the hepatic uptake of liposomes directly increases with liposome size in the sub-micron range (113, 114). This size-dependent uptake was proportional to the extent of opsonization, which increased with increase in liposome size from 200 nm to 800 nm with no enhancement of uptake at 200 nm (113). There was no effect of liposome size on the uptake of non-opsonized liposomes or liposomes incubated with serum heated at 56°C for 30 min or treated with anti-C3 antiserum (anti complement component 3), suggesting that, at the very least, the complement components in the alternative pathway were responsible for the opsonization (113, 114).

Although the above information is not directly related to the present study, it suggests that the magnitude of T cell responses might be liposome size-dependent. Until recently, there has been little information on the effects of liposome size on the T cell responses. Results of the size assessment study showed that SP1-020 specific T cell responses

were peptide dose-dependent rather than size-dependent. The reason for this has not been specifically investigated to date.

Contrary to an opsonin-dependent uptake explanation, substantial evidence has also been accumulated in support of an opsonin-independent uptake mechanism (173, 174). Using mouse liver as a model, it has been demonstrated that hepatic uptake of neutral or negatively-charged liposomes does not involve serum components because the data showed that there was no difference in the uptake between serum- and buffer-incubated liposomes (173). However, serum enhanced the liver uptake of positively-charged liposomes which caused aggregation with the negatively-charged serum proteins. It was likely that the aggregated liposomes were either trapped in the liver or taken up by Kupffer cells in the liver due to electrostatic-associated opsonins (173). Generally, liposomes containing negatively-charged phospholipids, such as phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidic acid (PA) have exhibited increased uptake compared to neutral liposomes. However, the negative charge was not the sole determinant of uptake because the specific headgroup of the anionic phospholipid and charge density in the liposome bilayer also affected the uptake (174). The opsonin-independent uptake mechanism emphasizes the importance of liposome-surface characteristics, such as type of lipid headgroup and surface charge that can be directly recognized by phagocytic cells (173, 174). In agreement with the opsonin-independent uptake mechanism,

the size-independent T cell responses in BALB/c mice indicated that the uptake might depend mainly on the liposome surface characteristics.

Opsonin-dependent and -independent uptake mechanisms do not necessarily conflict because one maybe dominate over the other in different experimental systems. Different liposome uptake mechanisms involved in different animal species have been reported (175, 176). In mice, Kupffer cells appear to be capable of recognizing surface characteristics of liposomes without alteration by serum components whereas rat or human Kupffer cells have surface recognition only after alteration by serum components when results of liver perfusion experiments were compared with buffer. Apparently there are major differences in opsonization activity by serum components in different animal species. For instance mouse Kupffer cells recognized human or bovine serum incubated liposomes, as evidenced by an approximately 10-fold uptake compared to that from either buffer or mouse serum-incubated liposomes (175). Mouse serum may be unique in lacking opsonization activity for liposomes of certain compositions, i.e. PC/CHOL and PC/CHOL/PS. Human serum showed the highest opsonization activity for the liposomes tested, except those containing PEG-PE (175). Thus, serum opsonization activity varied significantly among different animal species. Opsonin-dependent uptake of liposomes has been shown in rats and human but opsonin-independent in mice (113, 114, 173, 175, 176).

Human MUC1 peptides induced Th1 immune responses with liposomes containing 1% MPLA in C57BL/6 mice. It would be interesting to know which subset of the homologous T cell response was induced. Results of non-detectable levels of IL-4 and significant amounts of IFN-γ also indicated that type 1 T cell responses was elicited by SP1-020 liposomes in Balb/c mice. Immunopotentiation towards a Th1 response again supports the contention that liposomes containing immunomodulator MPLA promote type 1 associated immune responses (151, 153).

Although the magnitude of the T cell response was not significantly influenced by liposomes composed of different phospholipid compositions, the encapsulation efficiency of DMPC liposomes was much lower than DPPC or DSPC liposomes. Therefore, DSPC or DPPC liposomes seem to be more optimal delivery vehicles for the MUC1 peptides.

V. CONCLUSIONS AND SUMMARY

Conclusions based on the work described in this thesis are:

1. Encapsulation efficiency of hydrophilic MUC1 peptide was significantly improved in charged liposomes under low ionic strength conditions. Similar to most of the hydrophilic and non-bilayer associating molecules, MUC1 peptide incorporation is dependent on the size of the aqueous compartments in liposomes. The improved MUC1 encapsulation efficiency is due to the enlarged aqueous compartments in charged liposomes as a result of electrical repulsion between lipid bilayers.

2. More than 75% of the hydrophilic and amphiphilic MUC1 peptides were retained in FAT MLVs of DMPC or DPPC or DSPC:CHOL:DMPG (3:1:0.25, mole ratio) under the conditions of: 1) incubation at 37°C in PBS and 100% mouse or human serum for 24 h; 2) storage at 4°C in PBS for at least 12 months.

3. Liposome surface expression of MUC1 specific epitopes occurred with N-terminal acylated MUC1 peptide (BLP25) but not with hydrophilic (BP24 or BP25) or C-terminal acylated peptide (BLP24) as determined by a MUC1 specific mAb B248.7R2. Most likely, the MUC1 epitope specific for B248.7R2 assumed different conformations following lipid bilayer insertion of peptide having acylated groups at both ends of the molecule. The type of epitope expressed at the liposome surface is determined by the nature of incorporation of the peptide.

4. The formulation of MUC1 peptides significantly influenced the magnitude of T cell responses. Strong and specific MUC1 T cell responses were elicited by liposome-associated rather than non-associated peptides, suggesting a requirement of peptide association for T cell proliferative responses at a peptide dose of 2 nmole per mouse. Weak and non-specific T cell responses were induced by DETOX adjuvant formulations. MUC1 liposomes likely exerted adjuvanticity by protecting the peptide from enzymatic degradation as well as being a vehicle to deliver associated peptide antigens to the immune system in such an effective way so that immunogenicity of MUC1 peptides is significantly enhanced.

5. The nature of peptide association with liposomes was not critical for the type and magnitude of T cell responses. Both masked and surface-exposed peptide formulations elicited strong and specific MUC1 T cell responses accompanied with high levels of IFN-γ with no evidence of IL-4. Thus, MUC1 liposomes containing 1% MPLA elicited type 1 T cell responses independent of peptide location in liposomes. However, the mode of association significantly influenced humoral immune response. Only surface-exposed MUC1 liposomes induced high levels of anti-MUC1 antibodies dominated by IgG2b along with significant levels of IFN-γ, indicating type 1 associated humoral immune response. Besides the requirement of surface exposure of MUC1 epitopes, anti-MUC1 IgG production was also dependent on specific MUC1 T cell responses.

Results support the hypothesis that liposomes differing in antigen location may preferentially activate different immune pathways, namely, cellular and humoral pathways, by activating different types of antigen presenting cells. B lymphocytes can only be activated via antigen-specific Ig receptors by binding MUC1 epitopes expressed at liposome surfaces, whereas dendritic cells and macrophages are activated by uptake of MUC1 containing liposomes independent of the nature of peptide association.

6. Homologous mouse MUC1 specific T cell responses, as evaluated within a practical dose dependent range, were independent of phospholipid composition (DMPC or DPPC or DSPC:CHOL:DMPG, 3:1:0.25, mole ratio) and liposome size (120-900 nm in diameter) in BALB/c mice. Over the peptide dose range studied, specific human MUC1 T cell responses did not exhibit dose-dependency.

Brief summary of the thesis:

Strong and MUC1 specific type 1 associated immune responses were effectively induced by MUC1 liposomes containing MPLA. MUC1 peptide formulations profoundly influenced both cellular and humoral immune responses. A liposomal delivery system can be tailored to preferentially induce a cellular and/or a humoral immune response. Without using protein carriers and other strong adjuvants (i.e. RIBI's or Freund's), high levels of anti-MUC1 antibodies were generated by

surface-exposed MUC1 formulations. For the first time, strong and specific homologous T cell responses were demonstrated. Optimal formulation characteristics of MUC1 liposomes have also been determined. The results of this study are expected to advance knowledge on vaccine design.

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APPENDIX

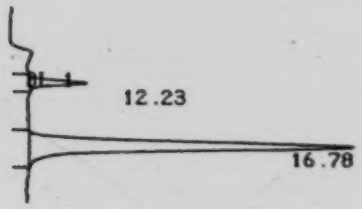

Figure A-1. Elution profile of BP24 in PBS (RT: retention time, 12.23 min) with internal reference phenol (RT, 16.78 min) on C-18 reversed phase radial-pak cartridge (8 mm x 100 mm). Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (96% to 54% A in 14 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

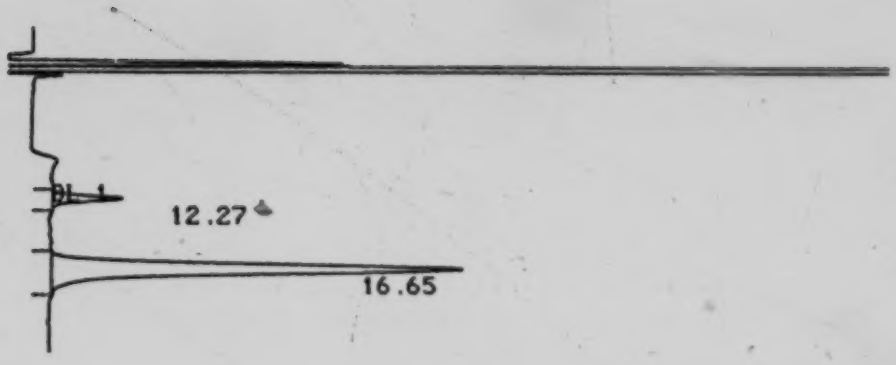

Figure A-2. Elution profile of BP24 (RT, 12.27 min) spiked with empty liposomes dissolved in 20% (v / v) absolute ethanol and internal reference phenol (RT, 16.65 min) on C-18 reversed phase radial-pak cartridge (8 mm x 100 mm). Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (96% to 54% A in 14 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

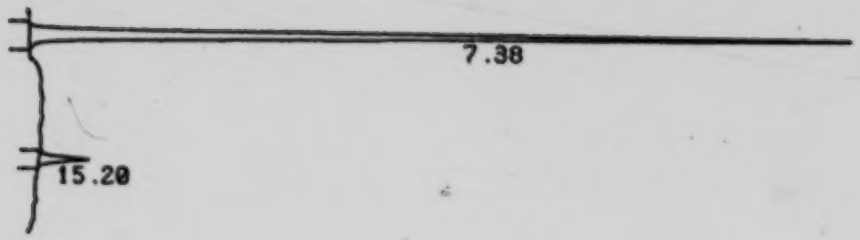

Figure A-3. Elution profile of BLP25 in PBS (RT, 15.20 min) with internal reference phenol (RT, 7.38 min) on C-18 reversed phase radial-pak cartridge (8 mm x 100 mm). Conditions: eluent A, 10% acetonitrile and eluent B, 70% acetonitrile; linear AB gradient (81% to 39% A in 14 min); flow rate, 1 ml / min; UV absorbance, 210 nm.

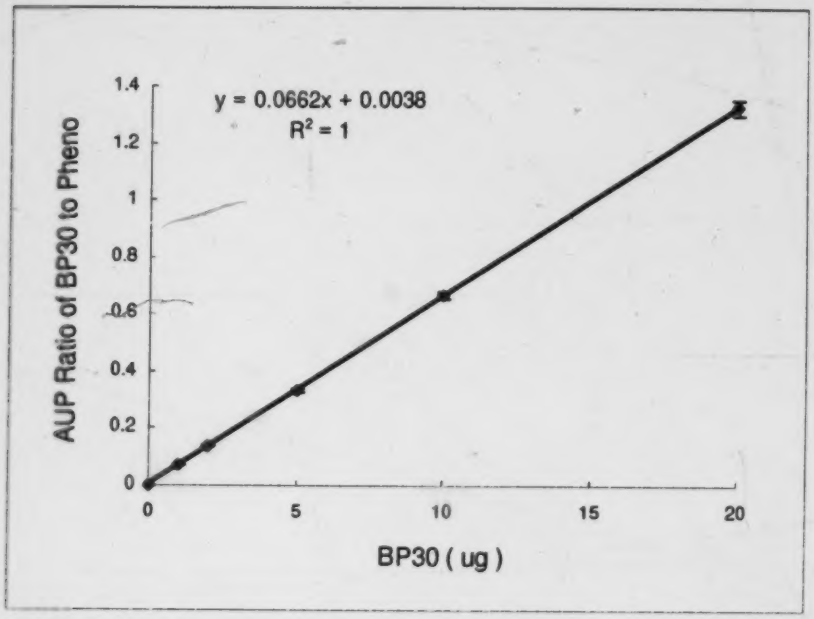

Figure B-1. Calibration curve of BP30 by HPLC method. AUP stands for area under the peak. Phenol was used as the internal reference. Values are expressed as means ± range, n=3.

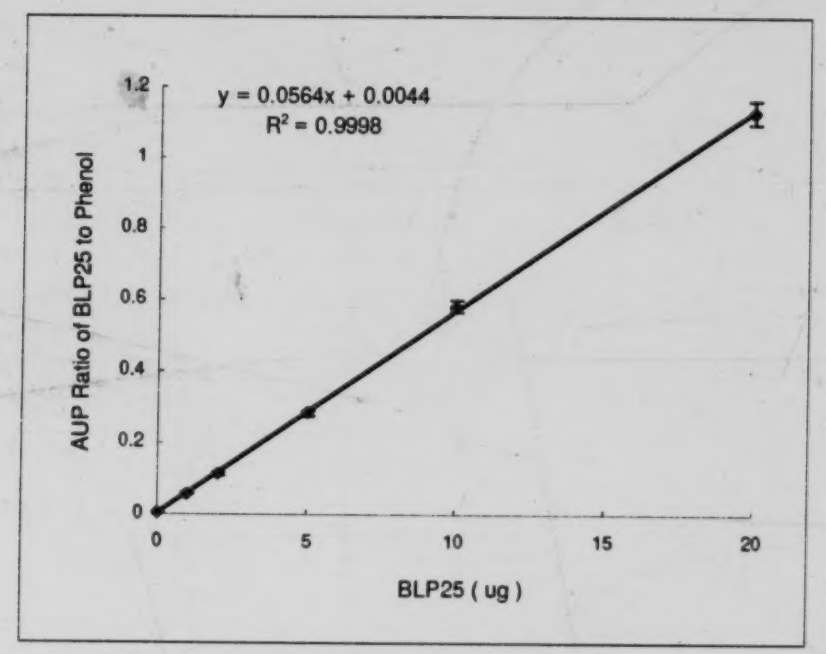

Figure B-2. Calibration curve of BLP25 by HPLC method. AUP stands for area under the peak. Phenol was used as the internal reference. Values are expressed as means ± range, n=3.

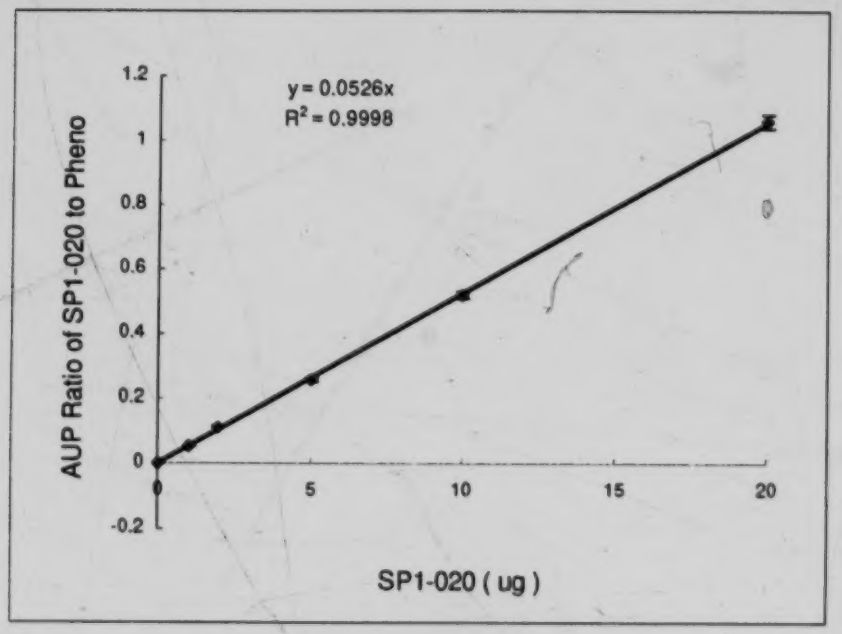

Figure B-3. Calibration curve of SP1-020 by HPLC method. AUP stands for area under the peak. Phenol was used as the internal reference. Values are expressed as means ± range, n=3.

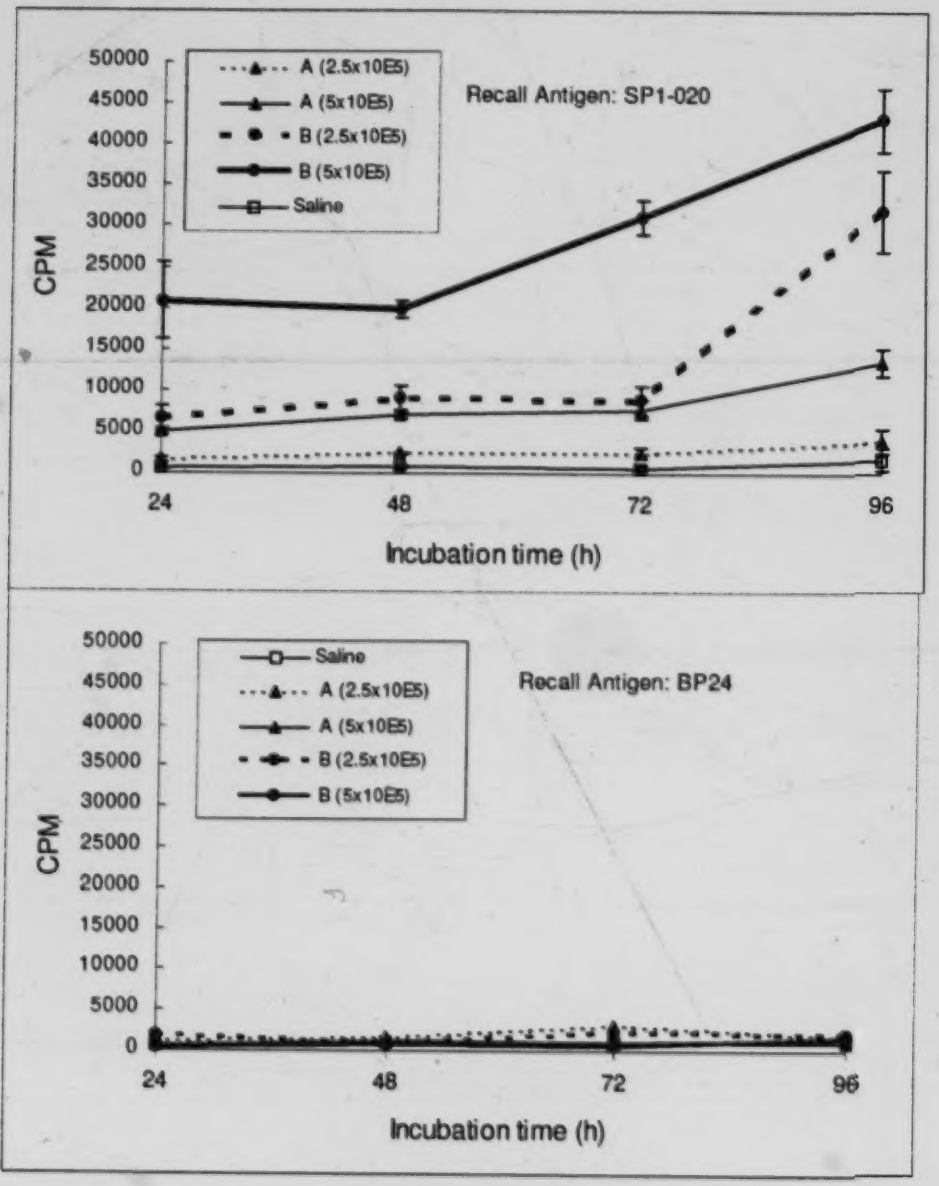

Figure C-1. T cell responses as a function of incubation time of T cell cultures. CAF1 mice were immunized with saline alone or SP1-020 liposomes of peptide dose of 4.5 μg or 92 μg per mouse (shown as A or B, respectively). T cells ($2.5x10^5$ / well or $5x10^5$ / well) purified from the popliteal lymph nodes on day 10 after immunization were cultured along with APCs in presence of SP1-020 (upper) or BP24 (below).

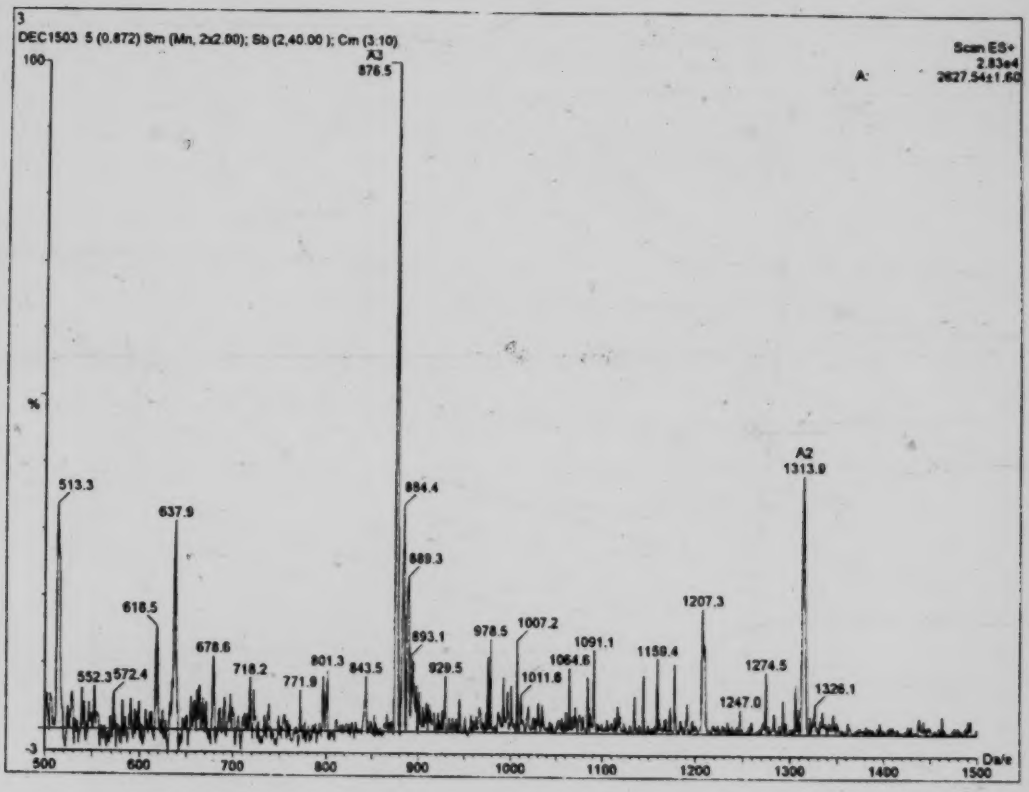

Figure D-1. Mass spectrum (at 79°C and 46 voltage) of BLP25 collected from HPLC pellet analyses of BLP25 liposomes incubated for 3 h with 100% human serum at 37°C. The molecular weight of BLP25 (A: 2627.54 ± 1.60) was generated based on the molecules carrying 2 charges (A2: 1313.9) and 3 charges (A3: 876.5) as shown.

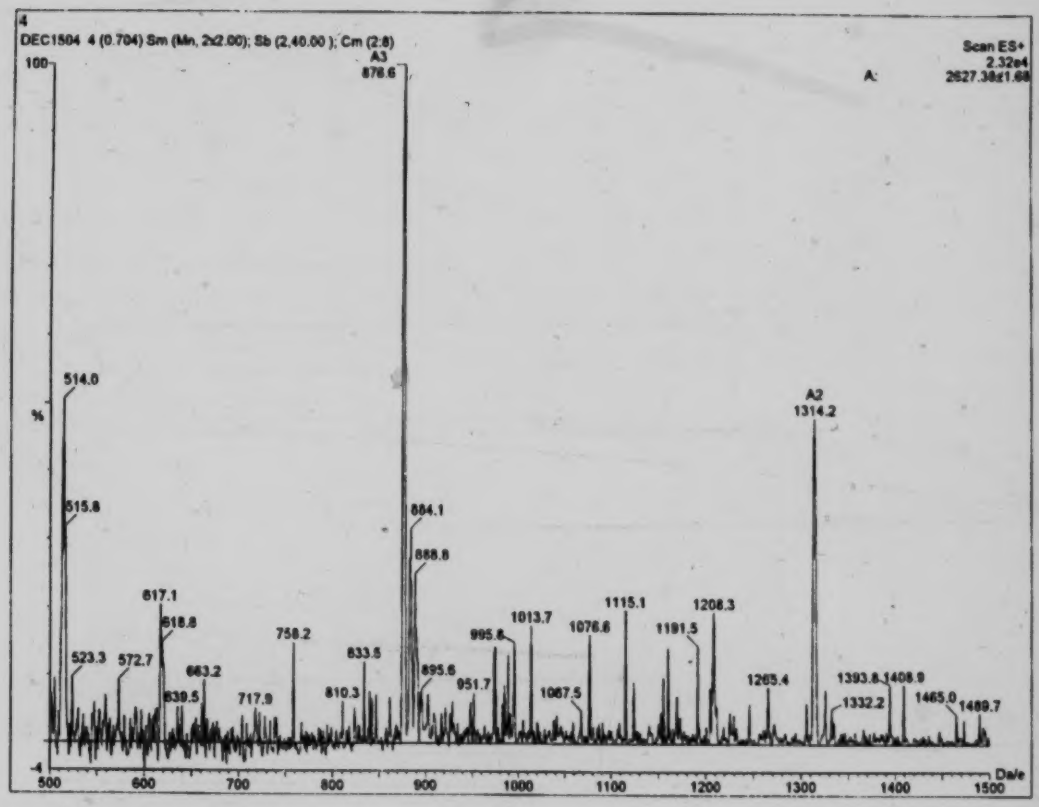

Figure D-2. Mass spectrum (at 79°C and 46 voltage) of BLP25 collected from HPLC pellet analyses of BLP25 liposomes incubated for 6 h with 100% human serum at 37°C. The molecular weight of BLP25 (A: 2627.38 ± 1.68) was generated based on the molecules carrying 2 charges (A2: 1314.2) and 3 charges (A3: 876.6) as shown.

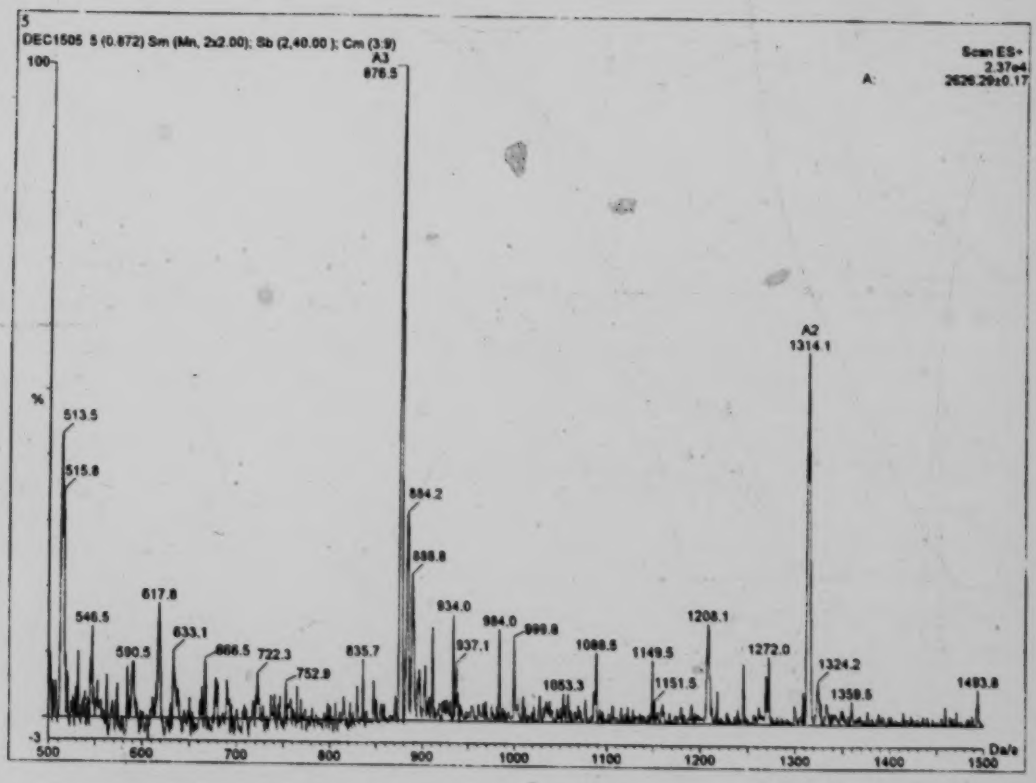

Figure D-3. Mass spectrum (at 79°C and 46 voltage) of BLP25 collected from HPLC pellet analyses of BLP25 liposomes incubated for 18 h with 100% human serum at 37°C. The molecular weight of BLP25 (A: 2626.29 ± 0.17) was generated based on the molecules carrying 2 charges (A2: 1314.1) and 3 charges (A3: 876.5) as shown.tively). T cells ($2.5x10^5$ / well or $5x10^5$ / well) purified from the popliteal lymph nodes on day 10 after immunization were cultured along with APCs in the presence of SP1-020 (upper) or BP24 (below).